As an alternative approach for receptor identification, a labeled albumin fusion protein can be photoaffinity linked with cell membrane or extract preparations that express the receptor molecule for the Therapeutoc protein component of an albumin fusion protein of the invention, the linked material may be resolved by PAGE analysis and exposed to X-ray film. The labeled complex containing the receptors of the fusion protein can be excised, resolved into peptide fragments, and subjected to protein microsequencing. The amino acid sequence obtained from microsequencing would be used to design a set of degenerate oligonucleotide probes to screen a cDNA library to identify the genes encoding the putative receptors.

Moreover, the techniques of gene-shuffling, motif-shuffling, exon-shuffling, and/or codon-shuffling (collectively referred to as "DNA shuffling") may be employed to modulate the activities of the fusion protein, and/or Therapeutic protein portion or albumin component of an albumin fusion protein of the present invention, thereby effectively generating agonists and antagonists of an albumin fusion protein of the present invention. *See generally*, U.S. Patent Nos. 5,605,793, 5,811,238, 5,830,721, 5,834,252, and 5,837,458, and Patten, P. A., *et al.*, *Curr. Opinion Biotechnol.* 8:724-33 (1997); Harayama, S. *Trends Biotechnol.* 16(2):76-82 (1998); Hansson, L. O., *et al.*, *J. Mol. Biol.* 287:265-76 (1999); and Lorenzo, M. M. and Blasco, R. *Biotechniques* 24(2):308-13 (1998); each of these patents and publications are hereby incorporated by reference). In one embodiment, alteration of polynucleotides encoding albumin fusion proteins of the invention and thus, the albumin fusion proteins encoded thereby, may be achieved by DNA shuffling. DNA shuffling involves the assembly of two or more DNA segments into a desired molecule by homologous, or site-specific, recombination. In another embodiment, polynucleotides encoding albumin fusion proteins of the invention and thus, the albumin fusion proteins encoded thereby, may be altered by being subjected to random mutagenesis by error-prone PCR, random nucleotide insertion or other methods prior to recombination. In another embodiment, one or more components, motifs, sections, parts, domains, fragments, etc., of an albumin fusion protein of the present invention may be recombined with one or more components, motifs, sections, parts, domains, fragments, etc. of one or more heterologous molecules. In preferred embodiments, the heterologous molecules are family members. In further preferred embodiments, the heterologous molecule is a growth factor such as, for example, platelet-derived growth factor (PDGF), insulin-like growth factor (IGF-I), transforming growth factor (TGF)-alpha, epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-beta, bone morphogenetic protein (BMP)-2, BMP-4, BMP-5, BMP-6, BMP-7, activins A and B, decapentaplegic(dpp), 60A, OP-2, dorsalin, growth differentiation factors (GDFs), nodal, MIS, inhibin-alpha, TGF-beta1, TGF-beta2, TGF-beta3, TGF-beta5, and glial-derived neurotrophic factor (GDNF).

Other preferred fragments are biologically active fragments of the Therapeutic protein portion and/or albumin component of the albumin fusion proteins of the present invention. Biologically active fragments are those exhibiting activity similar, but not necessarily identical, to an activity of a Therapeutic protein portion and/or albumin component of the albumin fusion proteins of the present invention. The biological activity of the fragments may include an improved desired activity, or a decreased undesirable activity.

Additionally, this invention provides a method of screening compounds to identify those which modulate the action of an albumin fusion protein of the present invention. An example of such an assay comprises combining a mammalian fibroblast cell, an albumin fusion protein of the present invention, and the compound to be screened and ^{3}[H] thymidine under cell culture conditions where the fibroblast cell would normally proliferate. A control assay may be performed in the absence of the compound to be screened and compared to the amount of fibroblast proliferation in the presence of the compound to determine if the compound stimulates proliferation by determining the uptake of ^{3}[H] thymidine in each case. The amount of fibroblast cell proliferation is measured by liquid scintillation chromatography which measures the incorporation of ^{3}[H] thymidine. Both agonist and antagonist compounds may be identified by this procedure.

In another method, a mammalian cell or membrane preparation expressing a receptor for the Therapeutic protien component of a fusion protine of the invention is incubated with a labeled fusion protein of the present invention in the presence of the compound. The ability of the compound to enhance or block this interaction could then be measured. Alternatively, the response of a known second messenger system following interaction of a compound to be screened and the receptor is measured and the ability of the compound to bind to the receptor and elicit a second messenger response is measured to determine if the compound is a potential fusion protein. Such second messenger systems include but are not limited to, cAMP guanylate cyclase, ion channels or phosphoinositide hydrolysis.

All of these above assays can be used as diagnostic or prognostic markers. The molecules discovered using these assays can be used to treat disease or to bring about a particular result in a patient (e.g., blood vessel growth) by activating or inhibiting the fusion protein/molecule. Moreover, the assays can discover agents which may inhibit or enhance the production of the albumin fusion proteins of the invention from suitably manipulated cells or tissues.

Therefore, the invention includes a method of identifying compounds which bind to an albumin fusion protein of the invention comprising the steps of: (a) incubating a candidate binding compound with an albumin fusion protein of the present invention; and (b) determining if binding has occurred. Moreover, the invention includes a method of

identifying agonists/antagonists comprising the steps of: (a) incubating a candidate compound with an albumin fusion protein of the present invention, (b) assaying a biological activity, and (b) determining if a biological activity of the fusion protein has been altered.

Targeted Delivery

In another embodiment, the invention provides a method of delivering compositions to targeted cells expressing a receptor for a component of an albumin fusion protein of the invention.

As discussed herein, fusion proteins of the invention may be associated with heterologous polypeptides, heterologous nucleic acids, toxins, or prodrugs via hydrophobic, hydrophilic, ionic and/or covalent interactions. In one embodiment, the invention provides a method for the specific delivery of compositions of the invention to cells by administering fusion proteins of the invention (including antibodies) that are associated with heterologous polypeptides or nucleic acids. In one example, the invention provides a method for delivering a Therapeutic protein into the targeted cell. In another example, the invention provides a method for delivering a single stranded nucleic acid (e.g., antisense or ribozymes) or double stranded nucleic acid (e.g., DNA that can integrate into the cell's genome or replicate episomally and that can be transcribed) into the targeted cell.

In another embodiment, the invention provides a method for the specific destruction of cells (e.g., the destruction of tumor cells) by administering an albumin fusion protein of the invention (e.g., polypeptides of the invention or antibodies of the invention) in association with toxins or cytotoxic prodrugs.

By "toxin" is meant compounds that bind and activate endogenous cytotoxic effector systems, radioisotopes, holotoxins, modified toxins, catalytic subunits of toxins, or any molecules or enzymes not normally present in or on the surface of a cell that under defined conditions cause the cell's death. Toxins that may be used according to the methods of the invention include, but are not limited to, radioisotopes known in the art, compounds such as, for example, antibodies (or complement fixing containing portions thereof) that bind an inherent or induced endogenous cytotoxic effector system, thymidine kinase, endonuclease, RNAse, alpha toxin, ricin, abrin, *Pseudomonas* exotoxin A, diphtheria toxin, saporin, momordin, gelonin, pokeweed antiviral protein, alpha-sarcin and cholera toxin. By "cytotoxic prodrug" is meant a non-toxic compound that is converted by an enzyme, normally present in the cell, into a cytotoxic compound. Cytotoxic prodrugs that may be used according to the methods of the invention include, but are not limited to, glutamyl derivatives of benzoic acid mustard alkylating agent, phosphate derivatives of etoposide or mitomycin C, cytosine arabinoside, daunorubisin, and phenoxyacetamide derivatives of doxorubicin.

Drug Screening

Further contemplated is the use of the albumin fusion proteins of the present invention, or the polynucleotides encoding these fusion proteins, to screen for molecules which modify the activities of the albumin fusion protein of the present invention or proteins corresponding to the Therapeutic protein portion of the albumin fusion protein. Such a method would include contacting the fusion protein with a selected compound(s) suspected of having antagonist or agonist activity, and assaying the activity of the fusion protein following binding.

This invention is particularly useful for screening therapeutic compounds by using the albumin fusion proteins of the present invention, or binding fragments thereof, in any of a variety of drug screening techniques. The albumin fusion protein employed in such a test may be affixed to a solid support, expressed on a cell surface, free in solution, or located intracellularly. One method of drug screening utilizes eukaryotic or prokaryotic host cells which are stably transformed with recombinant nucleic acids expressing the albumin fusion protein. Drugs are screened against such transformed cells or supernatants obtained from culturing such cells, in competitive binding assays. One may measure, for example, the formulation of complexes between the agent being tested and an albumin fusion protein of the present invention.

Thus, the present invention provides methods of screening for drugs or any other agents which affect activities mediated by the albumin fusion proteins of the present invention. These methods comprise contacting such an agent with an albumin fusion protein of the present invention or a fragment thereof and assaying for the presence of a complex between the agent and the albumin fusion protein or a fragment thereof, by methods well known in the art. In such a competitive binding assay, the agents to screen are typically labeled. Following incubation, free agent is separated from that present in bound form, and the amount of free or uncomplexed label is a measure of the ability of a particular agent to bind to the albumin fusion protein of the present invention.

Another technique for drug screening provides high throughput screening for compounds having suitable binding affinity to an albumin fusion protein of the present invention, and is described in great detail in European Patent Application 84/03564, published on September 13, 1984, which is incorporated herein by reference herein. Briefly stated, large numbers of different small peptide test compounds are synthesized on a solid substrate, such as plastic pins or some other surface. The peptide test compounds are reacted with an albumin fusion protein of the present invention and washed. Bound peptides are then detected

by methods well known in the art. Purified albumin fusion protein may be coated directly onto plates for use in the aforementioned drug screening techniques. In addition, non-neutralizing antibodies may be used to capture the peptide and immobilize it on the solid support.

This invention also contemplates the use of competitive drug screening assays in which neutralizing antibodies capable of binding an albumin fusion protein of the present invention specifically compete with a test compound for binding to the albumin fusion protein or fragments thereof. In this manner, the antibodies are used to detect the presence of any peptide which shares one or more antigenic epitopes with an albumin fusion protein of the invention.

Binding Peptides and Other Molecules

The invention also encompasses screening methods for identifying polypeptides and nonpolypeptides that bind albumin fusion proteins of the invention, and the binding molecules identified thereby. These binding molecules are useful, for example, as agonists and antagonists of the albumin fusion proteins of the invention. Such agonists and antagonists can be used, in accordance with the invention, in the therapeutic embodiments described in detail, below.

This method comprises the steps of:

contacting an albumin fusion protein of the invention with a plurality of molecules; and

identifying a molecule that binds the albumin fusion protein.

The step of contacting the albumin fusion protein of the invention with the plurality of molecules may be effected in a number of ways. For example, one may contemplate immobilizing the albumin fusion protein on a solid support and bringing a solution of the plurality of molecules in contact with the immobilized polypeptides. Such a procedure would be akin to an affinity chromatographic process, with the affinity matrix being comprised of the immobilized albumin fusion protein of the invention. The molecules having a selective affinity for the albumin fusion protein can then be purified by affinity selection. The nature of the solid support, process for attachment of the albumin fusion protein to the solid support, solvent, and conditions of the affinity isolation or selection are largely conventional and well known to those of ordinary skill in the art.

Alternatively, one may also separate a plurality of polypeptides into substantially separate fractions comprising a subset of or individual polypeptides. For instance, one can separate the plurality of polypeptides by gel electrophoresis, column chromatography, or like

method known to those of ordinary skill for the separation of polypeptides. The individual polypeptides can also be produced by a transformed host cell in such a way as to be expressed on or about its outer surface (e.g., a recombinant phage). Individual isolates can then be "probed" by an albumin fusion protein of the invention, optionally in the presence of an inducer should one be required for expression, to determine if any selective affinity interaction takes place between the albumin fusion protein and the individual clone. Prior to contacting the albumin fusion protein with each fraction comprising individual polypeptides, the polypeptides could first be transferred to a solid support for additional convenience. Such a solid support may simply be a piece of filter membrane, such as one made of nitrocellulose or nylon. In this manner, positive clones could be identified from a collection of transformed host cells of an expression library, which harbor a DNA construct encoding a polypeptide having a selective affinity for an albumin fusion protein of the invention. Furthermore, the amino acid sequence of the polypeptide having a selective affinity for an albumin fusion protein of the invention can be determined directly by conventional means or the coding sequence of the DNA encoding the polypeptide can frequently be determined more conveniently. The primary sequence can then be deduced from the corresponding DNA sequence. If the amino acid sequence is to be determined from the polypeptide itself, one may use microsequencing techniques. The sequencing technique may include mass spectroscopy.

In certain situations, it may be desirable to wash away any unbound polypeptides from a mixture of an albumin fusion protein of the invention and the plurality of polypeptides prior to attempting to determine or to detect the presence of a selective affinity interaction. Such a wash step may be particularly desirable when the albumin fusion protein of the invention or the plurality of polypeptides are bound to a solid support.

The plurality of molecules provided according to this method may be provided by way of diversity libraries, such as random or combinatorial peptide or nonpeptide libraries which can be screened for molecules that specifically bind an albumin fusion protein of the invention. Many libraries are known in the art that can be used, e.g., chemically synthesized libraries, recombinant (e.g., phage display libraries), and *in vitro* translation-based libraries. Examples of chemically synthesized libraries are described in Fodor et al., Science 251:767-773 (1991); Houghten et al., Nature 354:84-86 (1991); Lam et al., Nature 354:82-84 (1991); Medynski, Bio/Technology 12:709-710 (1994); Gallop et al., J. Medicinal Chemistry 37(9):1233-1251 (1994); Ohlmeyer et al., Proc. Natl. Acad. Sci. USA 90:10922-10926 (1993); Erb et al., Proc. Natl. Acad. Sci. USA 91:11422-11426 (1994); Houghten et al., Biotechniques 13:412 (1992); Jayawickreme et al., Proc. Natl. Acad. Sci. USA 91:1614-1618 (1994); Salmon et al., Proc. Natl. Acad. Sci. USA 90:11708-11712 (1993); PCT Publication No. WO 93/20242; and Brenner and Lerner, Proc. Natl. Acad. Sci. USA

89:5381-5383 (1992).

Examples of phage display libraries are described in Scott et al., Science 249:386-390 (1990); Devlin et al., Science, 249:404-406 (1990); Christian et al., 1992, J. Mol. Biol. 227:711-718 1992); Lenstra, J. Immunol. Meth. 152:149-157 (1992); Kay et al., Gene 128:59-65 (1993); and PCT Publication No. WO 94/18318 dated Aug. 18, 1994.

In vitro translation-based libraries include but are not limited to those described in PCT Publication No. WO 91/05058 dated Apr. 18, 1991; and Mattheakis et al., Proc. Natl. Acad. Sci. USA 91:9022-9026 (1994).

By way of examples of nonpeptide libraries, a benzodiazepine library (see e.g., Bunin et al., Proc. Natl. Acad. Sci. USA 91:4708-4712 (1994)) can be adapted for use. Peptoid libraries (Simon et al., Proc. Natl. Acad. Sci. USA 89:9367-9371 (1992)) can also be used. Another example of a library that can be used, in which the amide functionalities in peptides have been permethylated to generate a chemically transformed combinatorial library, is described by Ostresh et al. (Proc. Natl. Acad. Sci. USA 91:11138-11142 (1994)).

The variety of non-peptide libraries that are useful in the present invention is great. For example, Ecker and Crooke (Bio/Technology 13:351-360 (1995) list benzodiazepines, hydantoins, piperazinediones, biphenyls, sugar analogs, beta-mercaptoketones, arylacetic acids, acylpiperidines, benzopyrans, cubanes, xanthines, aminimides, and oxazolones as among the chemical species that form the basis of various libraries.

Non-peptide libraries can be classified broadly into two types: decorated monomers and oligomers. Decorated monomer libraries employ a relatively simple scaffold structure upon which a variety functional groups is added. Often the scaffold will be a molecule with a known useful pharmacological activity. For example, the scaffold might be the benzodiazepine structure.

Non-peptide oligomer libraries utilize a large number of monomers that are assembled together in ways that create new shapes that depend on the order of the monomers. Among the monomer units that have been used are carbamates, pyrrolinones, and morpholinos. Peptoids, peptide-like oligomers in which the side chain is attached to the alpha amino group rather than the alpha carbon, form the basis of another version of non-peptide oligomer libraries. The first non-peptide oligomer libraries utilized a single type of monomer and thus contained a repeating backbone. Recent libraries have utilized more than one monomer, giving the libraries added flexibility.

Screening the libraries can be accomplished by any of a variety of commonly known methods. See, e.g., the following references, which disclose screening of peptide libraries: Parmley et al., Adv. Exp. Med. Biol. 251:215-218 (1989); Scott et al,. Science 249:386-390 (1990); Fowlkes et al., BioTechniques 13:422-427 (1992); Oldenburg et al., Proc. Natl.

Acad. Sci. USA 89:5393-5397 (1992); Yu et al., Cell 76:933-945 (1994); Staudt et al., Science 241:577-580 (1988); Bock et al., Nature 355:564-566 (1992); Tuerk et al., Proc. Natl. Acad. Sci. USA 89:6988-6992 (1992); Ellington et al., Nature 355:850-852 (1992); U.S. Pat. No. 5,096,815, U.S. Pat. No. 5,223,409, and U.S. Pat. No. 5,198,346, all to Ladner et al.; Rebar et al., Science 263:671-673 (1993); and PCT Publication No. WO 94/18318.

In a specific embodiment, screening to identify a molecule that binds an albumin fusion protein of the invention can be carried out by contacting the library members with an albumin fusion protein of the invention immobilized on a solid phase and harvesting those library members that bind to the albumin fusion protein. Examples of such screening methods, termed "panning" techniques are described by way of example in Parmley et al., Gene 73:305-318 (1988); Fowlkes et al., BioTechniques 13:422-427 (1992); PCT Publication No. WO 94/18318; and in references cited herein.

In another embodiment, the two-hybrid system for selecting interacting proteins in yeast (Fields et al., Nature 340:245-246 (1989); Chien et al., Proc. Natl. Acad. Sci. USA 88:9578-9582 (1991) can be used to identify molecules that specifically bind to polypeptides of the invention.

Where the binding molecule is a polypeptide, the polypeptide can be conveniently selected from any peptide library, including random peptide libraries, combinatorial peptide libraries, or biased peptide libraries. The term "biased" is used herein to mean that the method of generating the library is manipulated so as to restrict one or more parameters that govern the diversity of the resulting collection of molecules, in this case peptides.

Thus, a truly random peptide library would generate a collection of peptides in which the probability of finding a particular amino acid at a given position of the peptide is the same for all 20 amino acids. A bias can be introduced into the library, however, by specifying, for example, that a lysine occur every fifth amino acid or that positions 4, 8, and 9 of a decapeptide library be fixed to include only arginine. Clearly, many types of biases can be contemplated, and the present invention is not restricted to any particular bias. Furthermore, the present invention contemplates specific types of peptide libraries, such as phage displayed peptide libraries and those that utilize a DNA construct comprising a lambda phage vector with a DNA insert.

As mentioned above, in the case of a binding molecule that is a polypeptide, the polypeptide may have about 6 to less than about 60 amino acid residues, preferably about 6 to about 10 amino acid residues, and most preferably, about 6 to about 22 amino acids. In another embodiment, a binding polypeptide has in the range of 15-100 amino acids, or 20-50 amino acids.

The selected binding polypeptide can be obtained by chemical synthesis or recombinant expression.

Other Activities

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention, may be employed in treatment for stimulating re-vascularization of ischemic tissues due to various disease conditions such as thrombosis, arteriosclerosis, and other cardiovascular conditions. The albumin fusion proteins of the invention and/or polynucleotides encoding albumin fusion proteins of the invention may also be employed to stimulate angiogenesis and limb regeneration, as discussed above.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed for treating wounds due to injuries, burns, post-operative tissue repair, and ulcers since they are mitogenic to various cells of different origins, such as fibroblast cells and skeletal muscle cells, and therefore, facilitate the repair or replacement of damaged or diseased tissue.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed stimulate neuronal growth and to treat and prevent neuronal damage which occurs in certain neuronal disorders or neuro-degenerative conditions such as Alzheimer's disease, Parkinson's disease, and AIDS-related complex. An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may have the ability to stimulate chondrocyte growth, therefore, they may be employed to enhance bone and periodontal regeneration and aid in tissue transplants or bone grafts.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be also be employed to prevent skin aging due to sunburn by stimulating keratinocyte growth.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed for preventing hair loss, since FGF family members activate hair-forming cells and promotes melanocyte growth. Along the same lines, an albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be employed to stimulate growth and differentiation of hematopoietic cells and bone marrow cells when used in combination with other cytokines.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be employed to maintain organs before transplantation or for supporting cell culture of primary tissues. An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may

also be employed for inducing tissue of mesodermal origin to differentiate in early embryos.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also increase or decrease the differentiation or proliferation of embryonic stem cells, besides, as discussed above, hematopoietic lineage.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be used to modulate mammalian characteristics, such as body height, weight, hair color, eye color, skin, percentage of adipose tissue, pigmentation, size, and shape (e.g., cosmetic surgery). Similarly, an albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be used to modulate mammalian metabolism affecting catabolism, anabolism, processing, utilization, and storage of energy.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may be used to change a mammal's mental state or physical state by influencing biorhythms, caricadic rhythms, depression (including depressive disorders), tendency for violence, tolerance for pain, reproductive capabilities (preferably by Activin or Inhibin-like activity), hormonal or endocrine levels, appetite, libido, memory, stress, or other cognitive qualities.

An albumin fusion protein of the invention and/or polynucleotide encoding an albumin fusion protein of the invention may also be used as a food additive or preservative, such as to increase or decrease storage capabilities, fat content, lipid, protein, carbohydrate, vitamins, minerals, cofactors or other nutritional components.

The above-recited applications have uses in a wide variety of hosts. Such hosts include, but are not limited to, human, murine, rabbit, goat, guinea pig, camel, horse, mouse, rat, hamster, pig, micro-pig, chicken, goat, cow, sheep, dog, cat, non-human primate, and human. In specific embodiments, the host is a mouse, rabbit, goat, guinea pig, chicken, rat, hamster, pig, sheep, dog or cat. In preferred embodiments, the host is a mammal. In most preferred embodiments, the host is a human.

Having generally described the invention, the same will be more readily understood by reference to the following examples, which are provided by way of illustration and are not intended as limiting.

Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the alterations detected in the present invention and practice the claimed methods. The following

working examples therefore, specifically point out preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

EXAMPLES

Example 1: Preparation of HA-hGH Fusion Proteins

An HA-hGH fusion protein was prepared as follows:

Cloning of hGH cDNA

The hGH cDNA was obtained from a human pituitary gland cDNA library (catalogue number HL1097v, Clontech Laboratories, Inc) by PCR amplification. Two oligonucleotides suitable for PCR amplification of the hGH cDNA, HGH1 and HGH2, were synthesized using an Applied Biosystems 380B Oligonucleotide Synthesizer.

HGH1: 5' - CCCAAGAATTCCCTTATCCAGGC - 3' (SEQ ID NO: 1)

HGH2: 5' - GGGAAGCTTAGAAGCCACAGGATCCCTCCACAG - 3' (SEQ ID NO: 2)

HGH 1 and HGH2 differed from the equivalent portion of the hGH cDNA sequence (Martial *et. al.*, 1979) by two and three nucleotides, respectively, such that after PCR amplification an *Eco*RI site would be introduced to the 5' end of the cDNA and a *Bam*H1 site would be introduced into the 3' end of the cDNA. In addition, HGH2 contained a *Hind*III site immediately downstream of the hGH sequence.

PCR amplification using a Perkin-Elmer-Cetus Thermal Cycler 9600 and a Perkin-Elmer-Cetus PCR kit, was performed using single-stranded DNA template isolated from the phage particles of the cDNA library as follows: 10 µL phage particles were lysed by the addition of 10 µL phage lysis buffer (280 µg/mL proteinase K in TE buffer) and incubation at 55°C for 15 min followed by 85°C for 15 min. After a 1 min. incubation on ice, phage debris was pelleted by centrifugation at 14,000 rpm for 3 min. The PCR mixture contained 6 µL of this DNA template, 0.1 µM of each primer and 200 µM of each deoxyribonucleotide. PCR was carried out for 30 cycles, denaturing at 94°C for 30 s, annealing at 65°C for 30 s and extending at 72°C for 30 s, increasing the extension time by 1 s per cycle.

Analysis of the reaction by gel electrophoresis showed a single product of the expected size (589 base pairs).

The PCR product was purified using Wizard PCR Preps DNA Purification System (Promega Corp) and then digested with *Eco*RI and *Hind*III. After further purification of the *Eco*RI-*Hind*III fragment by gel electrophoresis, the product was cloned into pUC19 (GIBCO

BRL) digested with *Eco*RI and *Hind*III, to give pHGH1. DNA sequencing of the *Eco*RI *Hind*III region showed that the PCR product was identical in sequence to the hGH sequence (Martial *et al.*, 1979), except at the 5' and 3' ends, where the *Eco*RI and *Bam*HI sites had been introduced, respectively.

Expression of the hGH cDNA.

The polylinker sequence of the phagemid pBluescribe (+) (Stratagene) was replaced by inserting an oligonucleotide linker, formed by annealing two 75-mer oligonucleotides, between the *Eco*RI and *Hind*III sites to form pBST(+). The new polylinker included a unique *Not*I site.

The *Not*I HA expression cassette of pAYE309 (EP 431 880) comprising the PRBI promoter, DNA encoding the HA/MFα-1 hybrid leader sequence, DNA encoding HA and the ADH1 terminator, was transferred to pBST(+) to form pHA1. The HA coding sequence was removed from this plasmid by digestion with HindIII followed by religation to form pHA2.

Cloning of the hGH cDNA, as described in Example 1, provided the hGH coding region lacking the pro-hGH sequence and the first 8 base pairs (bp) of the mature hGH sequence. In order to construct an expression plasmid for secretion of hGH from yeast, a yeast promoter, signal peptide and the first 8 bp of the hGH sequence were attached to the 5' end of the cloned hGH sequence as follows: The *Hind*III-*Sfa*NI fragment from pHA 1 was attached to the 5' end of the *Eco*RI/*Hind*III fragment from pHGHI via two synthetic oligonucleotides, HGH3 and HGH4 (which can anneal to one another in such a way as to generate a double stranded fragment of DNA with sticky ends that can anneal with *Sfa*NIand *Eco*RI sticky ends):

HGH3: 5' - GATAAAGATTCCCAAC - 3' (SEQ ID NO: 3)

HGH4: 5' - AATTGTTGGGAATCTTT- 3' (SEQ ID NO: 4)

The HindIII fragment so formed was cloned into *Hind*III-digested pHA2 to make pHGH2, such that the hGH cDNA was positioned downstream of the PRBI promoter and HA/MFα-1 fusion leader sequence (see, International Publication No. WO 90/01063). The *Not*I expression cassette contained in pHGH2, which included the *ADH1* terminator downstream of the hGH cDNA, was cloned into *Not*I-digested pSAC35 (Sleep *et al.*, BioTechnology 8:42 (1990)) to make pHGH12. This plasmid comprised the entire 2 μm plasmid to provide replication functions and the LEU2 gene for selection of transformants.

pHGH12 was introduced into *S. cerevisiae* D88 by transformation and individual transformants were grown for 3 days at 30°C in 10 mL YEPD (1% w/v yeast extract, 2 % w/v, peptone, 2 % w/v, dextrose).

After centrifugation of the cells, the supernatants were examined by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and were found to contain protein which was of the expected size and which was recognized by anti-hGH antiserum (Sigma, Poole, UK) on Western blots.

Cloning and expression of an HA-hGH fusion protein.

In order to fuse the *HA* cDNA to the 5' end of the hGH cDNA, the pHA1 *Hind*III-*Bsu*361 fragment (containing most of the *HA* cDNA) was joined to the pHGH1 *Eco*RI-*Hind*III fragment (containing most of the hGH cDNA) via two oligonucleotides, HGH7 and HGH8

HGH7: 5' - TTAGGCTTATTCCCAAC 3' (SEQ ID NO: 5)

HGH8: 5' - AATTGTTGGGAATAAGCC 3' (SEQ ID NO: 6)

The *Hind*III fragment so formed was cloned into pHA2 digested with *Hind*III to make pHGH10, and the *Not*I expression cassette of this plasmid was cloned into *Not*I-digested pSAC35 to make pHGH16.

pHGH16 was used to transform *S. cerevisiae* D88 and supernatants of cultures were analyzed as described above. A predominant band was observed that had a molecular weight of approximately 88 kD, corresponding to the combined masses of HA and hGH. Western blotting using anti-*HA* and anti-hGH antisera (Sigma) confirmed the presence of the two constituent parts of the albumin fusion protein.

The albumin fusion protein was purified from culture supernatant by cation exchange chromatography, followed by anion exchange and gel permeation chromatography. Analysis of the N-terminus of the protein by amino acid sequencing confirmed the presence of the expected albumin sequence.

An *in vitro* growth hormone activity assay (Ealey *et al.*, Growth Regulation 5:36 (1995)) indicated that the albumin fusion protein possessed full hGH activity. In a hypophysectomised rat weight gain model, performed essentially as described in the European Pharmacopoeia (1987, monograph 556), the fusion molecule was more potent than hGH when the same number of units of activity (based on the above *in vitro* assay) were administered daily. Further experiments in which the albumin fusion protein was administered once every four days showed a similar overall growth response to a daily administration of hGH. Pharmacokinetic experiments in which ^{125}I- labeled protein was administered to rats indicated an approximately ten-fold increase in circulatory half-life for the albumin fusion protein compared to hGH.

A similar plasmid was constructed in which DNA encoding the *S. cerevisiae* invertase (SUC2) leader sequence replaced the sequence for the hybrid leader, such that the encoded

leader and the junction (↓) with the HA sequence were as follows:

... MLLQAFLFLLAGFAAKISA ↓ DAHKS ..... (SEQ ID NO: 7) Invertase leader

HA sequence ...

On introduction into *S. cerevisiae* DBI, this plasmid directed the expression and secretion of the albumin fusion protein at a level similar to that obtained with pHGH16. Analysis of the N-terminus of the albumin fusion protein indicated precise and efficient cleavage of the leader sequence from the mature protein.

Cloning and expression of an hGH-HA fusion protein.

In order to fuse the hGH cDNA to the 5' end of the HA cDNA, the HA cDNA was first altered by site-directed mutagenesis to introduce an *Eco*NI site near the 5' end of the coding region. This was done by the method of Kunkel *et al.* (Methods in Enzymol. 154:367 (1987)) using single-stranded DNA template prepared from pHAI and a synthetic oligonucleotide, LEU4:

LEU4: 5' - GAGATGCACACCTGAGTGAGG - 3' (SEQ ID NO: 8)

Site-directed mutagenesis using this oligonucleotide changed the coding sequence of the HA cDNA from Lys4 to Leu4 (K4L). However, this change was repaired when the hGH cDNA was subsequently joined at the 5' end by linking the pHGH2 *Not*I-*Bam*HI fragment to the *Eco*NI-*Not*I fragment of the mutated pHAI, via the two oligonucleotides HGH5 and HGH6:

HGH5: 5' - GATCCTGTGGCTTCGATGCACACAAGA - 3' (SEQ ID NO: 9)

HGH6: 5' - CTCTTGTGTGCATCGAAGCCACAG - 3' (SEQ ID NO: 10)

The *Not*I fragment so formed was cloned into *Not*I-digested pSAC35 to make pHGH14. pHGH14 was used to transform S. cerevisiae D88 and supernatants of culture were analyzed as above. A predominant band was observed that had a molecular weight of approximately 88 kD, corresponding to the combined masses of hGH and HA. Western blotting using anti-HA and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion protein.

The albumin fusion protein was purified from culture supernatant by cation exchange chromatography, followed by anion exchange and gel permeation chromatography. Analysis of the N-terminus of the protein by amino acid sequencing confirmed the presence of the expected hGH sequence.

In vitro studies showed that the albumin fusion protein retained hGH activity, but was significantly less potent than an albumin fusion protein comprising full length HA (1-585) as

the N-terminal portion and hGH as the C-terminal portion, as described above.

Construction of plasmids for the expression of hGH fusions to domains of HA.

Fusion polypeptides were made in which the hGH molecule was fused to the first two domains of HA (residues 1 to 387). Fusion to the N terminus of hGH was achieved by joining the pHA1 *Hind*III-*Sap*I fragment, which contained most of the coding sequence for domains 1 and 2 of HA, to the pHGHI *Eco*RI-*Hind*III fragment, via the oligonucleotides HGH 11 and HGH 12:

HGH11: 5' - TGTGGAAGAGCCTCAGAATTTATTCCCAAC - 3' (SEQ ID NO: 11)

HGH12: 5' - AATTGTTGGGAATAAATTCTGAGGCTCTTCC - 3' (SEQ ID NO: 12)

The *Hind*III fragment so formed was cloned into *Hind*III-digested pHA2 to make pHGH37 and the *Not*I expression cassette of this plasmid was cloned into *Not*I-digested pSAC35.

The resulting plasmid, pHGH38, contained an expression cassette that was found to direct secretion of the fusion polypeptide into the supernatant when transformed into S. cerevisiae DB 1. Western blotting using anti-*HA* and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion protein.

The albumin fusion protein was purified from culture supernatant by cation exchange chromatography followed by gel permeation chromatography.

In vivo studies with purified protein indicated that the circulatory half-life was longer than that of hGH, and similar to that of an albumin fusion protein comprising full-length HA (1-585) as the N-terminal portion and hGH as the C-terminal portion, as described above. *In vitro* studies showed that the albumin fusion protein retained hGH activity.

Using a similar strategy as detailed above, an albumin fusion protein comprising the first domain of HA (residues 1-194) as the N-terminal portion and hGH as the C-terminal portion, was cloned and expressed in *S. cerevisiae* DBL. Western blotting of culture supernatant using anti-*HA* and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion protein.

Fusion of HA to hGH using a flexible linker sequence

Flexible linkers, comprising repeating units of $[Gly\text{-}Gly\text{-}Gly\text{-}Gly\text{-}Ser]_n$, where n was either 2 or 3, were introduced between the *HA* and hGH albumin fusion protein by cloning of the oligonucleotides HGH16, HGH17, HGH18 and HGH19:

HGH16:5'-TTAGGCTTAGGTGGCGGTGGATCCGGCGGTGGTGGATCTTTCCCA AC-3' (SEQ

ID NO: 13)

HGH17:5'-AATTGTTGGGAAAGATCCACCACCGCCGGATCCACCGCCACCTAAGCC-3' (SEQ ID NO: 14)

HGH18:5'-TTAGGCTTAGGCGGTGGTGGATCTGGTGGCGGCGGATCTGGTGGCGGTGGATCC TTCCCAAC-3' (SEQ ID NO: 15)

HGH19:5'-AATTGTTGGGAAGGATCCACCGCCACCAGATCCGCCGCCACCA GATCCACCACCGCCTAAGCC-3' (SEQ ID NO: 16)

Annealing of HGH16 with HGH17 resulted in n=2, while HGH18 annealed to HGH19 resulted in n=3. After annealing, the double- stranded oligonucleotides were cloned with the *Eco*RI-*Bsu*361 fragment isolated from pHGH1 into *Bsu*361-digested pHGH10 to make pHGH56 (where n=2) and pHGH57 (where n=3). The *Not*I expression cassettes from these plasmids were cloned into *Not*I-digested pSAC35 to make pHGH58 and pHGH59, respectively.

Cloning of the oligonucleotides to make pHGH56 and pHGH57 introduced a *Bam*HI site in the linker sequences. It was therefore possible to construct linker sequences in which n=1 and n = 4, by joining either the *Hind*III-*Bam*HI fragment from pHGH56 to the *Bam*HI-*Hind*III fragment from pHGH57 (making n = 1), or the *Hind*III-*Bam*HI fragment from pHGH57 to the *Bam*HI-*Hind*III fragment from pHGH56 (making n=2). Cloning of these fragments into the HindIII site of pHA2, resulted in pHGH60 (n= 1) and pHGH61 (n=4). The *Not*I expression cassettes from pHGH60 and pHGH61 were cloned into *Not*I-digested pSAC35 to make pHGH62 and pHGH63, respectively.

Transformation of *S. cerevisiae* with pHGH58, pHGH59, pHGH62 and pHGH63 resulted in transformants that secreted the fusion polypeptides into the supernatant. Western blotting using anti-*HA* and anti-hGH antisera confirmed the presence of the two constituent parts of the albumin fusion proteins.

The albumin fusion proteins were purified from culture supernatant by cation exchange chromatography, followed by anion exchange and gel permeation chromatography. Analysis of the N-termini of the proteins by amino acid sequencing confirmed the presence of the expected albumin sequence. Analysis of the purified proteins by electrospray mass spectrometry confirmed an increase in mass of 315 D (n=l), 630 D (n=2), 945 D (n=3) and 1260 D (n=4) compared to the *HA*-hGH fusion protein described above. The purified protein was found to be active *in vitro*.

Increased Shelf-Life of HA-hGH fusion proteins: Methods

HA-hGH and hGH were separately diluted in cell culture media containing 5% horse serum to final concentrations of 100-200 μg/ml and incubated at 4, 37 or 50°C. At time zero

and at weekly intervals thereafter, aliquots of the samples were tested for their biological activity in the Nb2 cell proliferation assay, and the data normalized to the biological activity of the control (hGH solution at time zero). In other assays hGH and HA-hGH were incubated in phosphate buffer saline in at 4, 37 and 50 degree C.

Nb2 cell proliferation assay: The growth of these cells is dependent on hGH or other lactogenic hormones. In a typical experiment 10^4 cells /well are plated in 96-well plate in the presence of different concentration of hGH or HA-hGH in media such as DMEM containing 5-10% horse serum for 24-48 hrs in the incubator. After the incubation period, 1:10 volume of MTT (5mg/ml in H_2O) is added to each well and the plate is incubated for a further 6-16 hrs. The growing cells convert MTT to insoluble formazan. The formazan is solublized by acidic isopropanol, and the color produced is measured at 570 nm on microtiter plate reader. The extent of formazan formation reflects the level of cellular proliferation.

Increased shelf-life of HA-hGH fusion proteins: Results

The fusion of Therapeutic proteins to albumin confers stability in aqueous or other solution. Figure 1 depicts the extended shelf-life of an HA fusion protein in terms of the biological activity of HA-hGH remaining after storage in cell culture media for up to 5 weeks at 37°C. A solution of 200 μg/ml HA-hGH was prepared in tissue culture media containing 5% horse serum, and the solution incubated at 37°C starting at time zero. At the indicated times, a sample was removed and tested for its biological activity in the Nb2 cell assay, at 2 ng/ml final concentration. As shown in Figure 1, the biological activity of HA-hGH remains essentially intact (within experimental variation) after 5 weeks of incubation at 37°C. The recombinant hGH used as control for this experiment lost its biological activity in the first week of the experiment.

Figure 2 shows the stability of HA-hGH after storage in cell culture media for up to 3 weeks at 4, 37, or 50°C. At time zero, a solution of HA-hGH was prepared in tissue culture media containing 5% horse serum, and incubated at 4, 37, and 50°C. At the indicated periods a sample was removed and assayed for its biological activity in the Nb2 cell proliferation assay, at 60 ng/ml final concentration. HA-hGH retains over 90% of its initial activity at all temperatures tested for at least 3 weeks after incubation while hGH loses its biological activity within the first week. This level of activity is further retained for at least 7 weeks at 37° C and 5 weeks at 50° C. These results indicate that HA-hGH is highly stable in aqueous solution even under temperature stress.

Figures 3A and 3B show the stable biological activity of HA-hGH compared to hGH in the Nb2 cell proliferation assay. Nb2 cells were grown in the presence of increasing

concentrations of recombinant hGH or HA-hGH, added at time zero. The cells were incubated for 24 or 48 hours before measuring the extent of proliferation by the MTT method. The increased stability of HA-hGH in the assay results in essentially the same proliferative activity at 24 hours (Figure 3A) as at 48 hours (Figure 3B) while hGH shows a significant reduction in its proliferative activity after 48 hours of incubation (Figures 3A and 3B). Compared to hGH, the HA-hGH has lower biological potency after 1 day; the albumin fusion protein is about 5 fold less potent than hGH. However, after 2 days the HA-hGH shows essentially the same potency as hGH due to the short life of hGH in the assay. This increase in the stability of the hGH as an albumin fusion protein has a major unexpected impact on the biological activity of the protein. Although the potency of the albumin fusion proteins is slightly lower than the unfused counterparts in rapid bioassays, their biological stability results in much higher biological activity in the longer term *in vitro* assay or *in vivo* assays.

Example 2: Preparation of HA-fusion proteins.

Figure 4 shows a map of a plasmid (pPPC0005) that can be used as the base vector for cloning the cDNAs of therapeutic partners to form HA-fusions. For example, digestion of this vector with the restriction enzymes *Bsu36I*/Partial *Hin*dIII will allow for the insertion of a cDNA modified at the 5' end to encode the last 5 amino acids of HA including the *Bsu36I* site and at the 3' end to include a double stop codon and *Hin*dIII site. As another example, digestion of this vector with the restriction enzymes *Bsu36I*, *SphI* allows for the insertion of a cDNA modified at the 5' end to encode the last 5 amino acids of HA including the *Bsu36I* site and at the 3' end to include a double stop codon, *Hin*dIII site and the *ADHI* terminator sequence up to and including the *SphI* site.

This plasmid may easily be modified by one of skill in the art, for example, to modify, add or delete restriction sites so that one may more easily clone a Therapeutic protein, or fragment or variant of into the vector for the purpose of making an albumin fusion protein of the invention.

For example, for the purpose of making an albumin fusion protein where the Therapeutic moiety is placed N-terminal to the (mature) albumin protein, restriction sites were added at the 5' end of the DNA encoding HA in pPPC0005 shown in Figure 4).

Because it was desired to add unique XhoI and ClaI sites at the 5' end of the DNA encoding the HA protein in pPPC0005, it was first necessary to remove those same sites from the plasmid (located 3' of the ADH1 terminator sequence). This was accomplished by cutting pPPC0005 with XhoI and ClaI, filling in the sticky ends with T4 DNA polymerase, and religating the blunt ends to create pPPC0006

Engineering the Xho and Cla I restriction sites into the Fusion leader sequence just 5' of the DNA encoding the HA protein in pPPC0006 was accomplished using two rounds of PCR. The first pair of oligonucleotides are those of SEQ ID NO:19 and SEQ ID NO:20. SEQ ID 19 contains four point mutations relative to the DNA sequence encoding the Fusion leaadr sequence and the beginning of the HA protein. These mutations are necessary to create the XhoI site in the fusion leader sequence and the Cla I site just at the beginning of the DNA encoding the HA protein. These four mutations are underlined in the sequence shown below. In pPPC0006 the nucleotides at these four positions from 5' to 3' are T, G, T, and G.

5'-GCCTCGAGAAAAGAGATGCACACAAGAGTGAGGTTGCTCATCGATTTAAAGAT TTGGG-3' (SEQ ID NO:19)

5'-AATCGATGAGCAACCTCACTCTTGTGTGCATCTCTTTTCTCGAGGCTCCTGGAA TAAGC-3' (SEQ ID NO:20). A second round of PCR is then performed with an upstream flanking primer, 5'-TACAAACTTAAGAGTCCAATTAGC-3' (SEQ ID NO:21) and a downstream flanking primer 5'-CACTTCTCTAGAGTGGTTTCATATGTCTT-3' (SEQ ID NO:22). The resulting PCR product is then purified and then digested with AflI and XbaI and ligated into the same sites in pPPC0006 creating pScCHSA. The resulting plasmid will have an XhoI sites engineered into the fusion leader sequence. The presence of the XhoI site creates a single amino acid change in the end of fusion leader sequence from LDKR to LEKR. The D to E change will not be present in the final albumin fusion protein expression plasmid if one ligates into the XhoI and Cla I sites a fragment comprising the Therapeutic moiety which has a 5' SalI sticky end (which is compatible with the XhoI end) and a 3' ClaI end. Ligation of the XhoI to the SalI restores the original amino acid sequence of the Fusion leader sequence. The Therapeutic protein moiety may be inserted after the Kex2 site (Kex2 claeves after the dibasic amino acid sequence KR at the end of the Fusion leader sequence) and before the ClaI site.

In addition, for the purpose of making an albumin fusion protein where the Therapeutic moiety is placed C-terminal to the (mature) albumin protein, four, eight-base-pair restriction sites were added at the 3' end of the DNA encoding HA in pScCHSA. As an example, it was felt to be desirable to incorporate AscI, FseI, and PmeI restriction sites in between the Bsu36I and HindIII sites at the end of the DNA encoding the HA protein in pScCHSA. This was accomplished through the use of two complementary synthetic oligonucleotides (SEQ ID NO:19 and SEQ ID NO:20) which contain the desired restriction sites.

5'-AAGCTGCCTTAGGCTTATAATAAGGCGCGCCGGCCGGCCGTTTAAACTAAGCT TAATTCT-3' (SEQ ID NO:23) and

5-AGAATTAAGCTTAGTTTAAACGGCCGGCCGGCGCGCCTTATTATAAGCCTAAGG

CAGCTT-3' (SEQ ID NO:24). These oligonucleotides may be annealed and digested with Bsu36I and HindIII and ligated into the same sites located at the end of the DNA encoding the HA protein in pScCHSA creating pScNHSA, using techniques known in the art.

Making vectors comprising albumin fusion proteins where the albumin moiety is N-terminal to the Therapeutic moiety.

The DNA encoding the Therapeutic moiety may be PCR amplified using primers that will add DNA encoding the last five amino acids of the HA (and containing the Bsu36I site) onto the 5' end of the DNA encoding a Therapeutic protein and a STOP codon and appropriate cloning sites onto the 3' end of the coding sequence. For instance, the forward primer used to amplify the DNA encoding a Therapeutic protein might have the sequence, 5'-aagctGCCTTAGGCTTA$(N)_{15}$-3' (SEQ ID NO:25) where the underlined sequence is a Bsu36I site, the upper case nucleotides encode the last four amino acids of the mature HA protein (ALGL), and $(N)_{15}$ is identical to the first 15 nucleotides encoding the Therapetic protein of interest. Similarly, the reverse primer used to amplify the DNA encoding a Therapeutic protein might have the sequence, 5'-GCGCGC*GTTTAAAC*GGCCGGCCGGCGCGCCTTATTA$(N)_{15}$-3' (SEQ ID NO:26) where the italicized nucleotides is a PmeI site, the double underlined nucleotides are a FseI site, the singly underlined text is a PmeI site, the boxed nucleotides are the reverse complement of two tandem stop codons, and $(N)_{15}$ is identical to the reverse complement of the last 15 nucleotides encoding the Therapeutic protein of interest. Once the PCR product is amplified it may be cut with Bsu36I and one of (AscI, FseI, or PmeI) and ligated into pScNHSA.

Making vectors comprising albumin fusion proteins where the albumin moiety is N-terminal to the Therapeutic moiety.

The DNA encoding the Therapeutic moiety may be PCR amplified using primers that will add DNA encoding the last three amino acids of the Fusion leader sequence (and containing a SalI site) onto the 5' end of the DNA encoding a Therapeutic protein and the first few amino acids of the HA (and containing a ClaI site. For instance, the forward primer used to amplify the DNA encoding a Therapeutic protein might have the sequence, 5'-aggagcgtcGACAAAAGA$(N)_{15}$-3' (SEQ ID NO:27) where the underlined sequence is a Sal I site, the upper case nucleotides encode the last three amino acids of the Fusion leader sequence (DKR), and $(N)_{15}$ is identical to the first 15 nucleotides encoding the Therapetic protein of interest. Similarly, the reverse primer used to amplify the DNA encoding a Therapeutic protein might have the sequence,

5'-CTTTAA*ATCGAT*GAGCAACCTCACTCTTGTGTGCATC$(N)_{15}$-3' (SEQ ID NO:28) where the italicized nucleotides are a ClaI site, the underlined nucleotides are the reverse complement of the DNA encoding the first 9 amino acids of HA (DAHKSEVAH), and $(N)_{15}$ is identical to the reverse complement of the last 15 nucleotides encoding the Therapeutic protein of interest. Once the PCR product is amplified it may be cut with SalI and ClaI and ligated into pScCHSA digested with XhoI and Cla I.

Expression of an Albumin Fusion Protein in yeast.

The Not I fragment containing the DNA encoding either an N-terminal or C-terminal albumin fusion protein generated from pScCHSA or pScNHSA may then be cloned in to the NotI site of pSAC35.

Expression of an Albumin Fusion Protein from Mammalian cell lines

The HSA gene has also been cloned into a the pC4 vector which is more suitable for mammalian culture systems creating plasmid pC4:HSA. More specifically, pC4HSA was generated by PCR amplifying the mature HSA gene with a 5' primer (SEQ ID NO:30) that anneals to the 5' end of DNA encoding the mature form of the HSA protein (e.g, DNA in plasmid pScCHSA),incorporates BamHI (Shown in italics below) and HindIII (shown singly underlined below) cloning sites, attaches a kozak sequence (shown double underlined below) and DNA encoding the natural HSA signal peptide (MKWVSFISLLFLFSSAYSRSLDKR, SEQ ID NO:29) (shown in bold below), and a 3' primer (SEQ ID NO:31) that anneals to the 3' end of DNA encoding the mature form of the HSA protein and incorporates an Asp718 restriction site (shown in bold below). The DNA encoding the natural human serum albumin leader sequence in SEQ ID NO:30 also contains a modification that introduces a XhoI site that is boxed below.

5'-TCAG*GGATCC*AAGCTTCCGCCACCATGAAGTGGGTAACCTTTATTTCCCTTCTTTTTCTCTTTAG

CTCGGCTTA CTCGAG GGGTGTGTTTCGTCGAGATGCACACAAGAGTGAG-3' (SEQ ID NO:30)

5"-GCAGCGGTACCGAATTCGGCGCGCCTTATAAGCCTAAGGCAGC-3' (SEQ ID NO:31)

This PCR product (1.85kb) is then purified and digested with BamHI and Asp718 and cloned into the same sites in pC4 (ATCC Accession No. 209646) to produce pC4:HSA

Making vectors comprising albumin fusion proteins where the albumin moiety is C-terminal to the Therapeutic moiety using the pC4:HSA vector

Using pC4:HSA, albumin fusion proteins in which the Therapeutic protein moiety is N terminal to the albumin sequence, one can clone DNA encoding a Therapeutic protein that has its own signal sequence between the Bam HI (or HindIII) and ClaI sites. When cloning into either the BamHI or Hind III site remember to include Kozak sequence (CCGCCACCATG) prior to translational start codon of DNA encoding the Therapeutic Protein to be subcloned. If the Therapeutic does not have a signal sequence, the DNA encoding that Therapeutic protein may be cloned in between the XhoI and ClaI sites. When using the XhoI site, the following 5' (SEQ ID NO:32) and 3' (SEQ IDNO:33) PCR primers may be used:

5'-CCGCCGCTCGAGGGGTGTGTTTCGTCGA$(N)_{18}$-3' (SEQ ID NO: 32)

5'-AGTCCCATCGATGAGCAACCTCACTCTTGTGTGCATC$(N)_{18}$-3' (SEQ ID NO:33)

In SEQ ID NO:32, the underlined sequence is an XhoI site; and the XhoI site and the DNA following the XhoI site encode for the last seven amino acids of the leader sequence of natural human serum albumin. In SEQ ID NO:33, the underlined sequence is a ClaI site; and the ClaI site and the DNA following it encode are the reverse complement of the DNA encoding the first 10 amino acids of themature HSA protein (SEQ ID NO:18). In SEQ ID NO:32 "$(N)_{18}$" is DNA identical to the first 18 nucleotides encoding the Therapeutic protein of interest.). In SEQ ID NO:33 "$(N)_{18}$" is the reverse complement of DNA encoding the last 18 nucleotides encoding the Therapeutic protein of interest. Using these two primers, one may PCR amplify the Therapeutic protein of interest, purify the PCR product, digest it with XhoI and ClaI restriction enzymes and then and clone it into the with XhoI and ClaI sites in the pC4:HSA vector.

Making vectors comprising albumin fusion proteins where the albumin moiety is N-terminal to the Therapeutic moiety using the pC4:HSA vector

Using pC4:HSA, albumin fusion proteins in which the Therapeutic protein moiety is N terminal to the albumin sequence, one can clone DNA encoding a Therapeutic protein between the Bsu36I and AscI restriction sites. When cloning into the Bsu36I and AscI, the same primer design used to clone in the yeast vector system (SEQ ID NO:25 and 26) may be employed.

The pC4 vector is especially suitable for expression of albumin fusion proteins from CHO cells. For expression, in other mammalian cell types, e.g., NSO cells, it may be useful to subclone the HindIII - EcoRI fragment containing the DNA encoding an albumin fusion protein (from a pC4 vector in which the DNA encoding the Therapeutic protein has already been cloned in frame with the DNA encoding (the mature form of) human serum albumin)

into another expression vector (such as any of the mammalian expression vectors described herein).

Example 3: Preparation of HA-cytokine or HA-growth factor fusion proteins (such as EPO, GMCSF, GCSF)

The cDNA for the cytokine or growth factor of interest, such as EPO, can be isolated by a variety of means including from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for all of these proteins are known and available, for instance, in U.S. Patents 4,703,008, 4,810,643 and 5,908,763. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. EPO (or other cytokine) cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines, a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 4: Preparation of HA-IFN fusion proteins (such as IFNα)

The cDNA for the interferon of interest such as IFNα can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for interferons, such as IFNα are known and available, for instance, in U.S. Patents 5,326,859 and 4,588,585, in EP 32 134, as well as in public databases such as GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used to clone the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus of the HA sequence, with or without the use of a spacer sequence. The IFNα (or other interferon) cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast (see Figure 8). The albumin fusion protein

secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Maximum protein recovery from vials

The albumin fusion proteins of the invention have a high degree of stability even when they are packaged at low concentrations. In addition, in spite of the low protein concentration, good fusion-protein recovery is observed even when the aqueous solution includes no other protein added to minimize binding to the vial walls. Figure 5 compares the recovery of vial-stored HA-IFN solutions with a stock solution. 6 or 30 μg/ml HA-IFN solutions were placed in vials and stored at 4°C. After 48 or 72 hrs a volume originally equivalent to 10 ng of sample was removed and measured in an IFN sandwich ELISA. The estimated values were compared to that of a high concentration stock solution. As shown, there is essentially no loss of the sample in these vials, indicating that addition of exogenous material such as albumin is not necessary to prevent sample loss to the wall of the vials

In vivo stability and bioavailability of HA-α-IFN fusions

To determine the in vivo stability and bioavailability of a HA-α-IFN fusion molecule, the purified fusion molecule (from yeast) was administered to monkeys at the dosages and time points described in Figures 6 and 7. Pharmaceutical compositions formulated from HA-α-IFN fusions may account for the extended serum half-life and bioavailability exemplified in Figures 6 and 7. Accordingly, pharmaceutical compositions may be formulated to contain lower dosages of alpha-interferon activity compared to the native alpha-interferon molecule.

Pharmaceutical compositions containing HA-α-IFN fusions may be used to treat or prevent disease in patients with any disease or disease state that can be modulated by the administration of α-IFN. Such diseases include, but are not limited to, hairy cell leukemia, Kaposi's sarcoma, genital and anal warts, chronic hepatitis B, chronic non-A, non-B hepatitis, in particular hepatitis C, hepatitis D, chronic myelogenous leukemia, renal cell carcinoma, bladder carcinoma, ovarian and cervical carcinoma, skin cancers, recurrent respirator papillomatosis, non-Hodgkin's and cutaneous T-cell lymphomas, melanoma, multiple myeloma , AIDS, multiple sclerosis, gliobastoma, etc. (see Interferon Alpha, In:

AHFS Drug Information, 1997.

Accordingly, the invention includes pharmaceutical compositions containing a HA-α-IFN fusion protein, polypeptide or peptide formulated with the proper dosage for human administration. The invention also includes methods of treating patients in need of such treatment comprising at least the step of administering a pharmaceutical composition containing at least one HA-α-IFN fusion protein, polypeptide or peptide.

Bifunctional HA-_-IFN fusions

The HA-α-IFN expression vector of Figure 8 is modified to include an insertion for the expression of bifunctional HA-α-IFN fusion proteins. For instance, the cDNA for a second protein of interest may be inserted in frame downstream of the "rHA-IFN" sequence after the double stop codon has been removed or shifted downstream of the coding sequence.

In one version of a bifunctional HA-α-IFN fusion protein, an antibody or fragment against B-lymphocyte stimulator protein (GenBank Acc 4455139) or polypeptide may be fused to one end of the HA component of the fusion molecule. This bifunctional protein is useful for modulating any immune response generated by the α-IFN component of the fusion.

Example 5: Preparation of HA-hormone fusion protein (such as insulin, LH, FSH)

The cDNA for the hormone of interest such as insulin can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for all of these proteins are known and available, for instance, in public databases such as GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The hormone cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This

expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 6: Preparation of HA-soluble receptor or HA-binding protein fusion protein such as HA-TNF receptor

The cDNA for the soluble receptor or binding protein of interest such as TNF receptor can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The nucleotide sequences for all of these proteins are known and available, for instance, in GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The receptor cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 7: Preparation of HA-growth factors such as HA-IGF-1 fusion protein

The cDNA for the growth factor of interest such as IGF-1 can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods (see GenBank Acc. No. NP_000609). The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The growth factor cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used

employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 8: Preparation of HA-single chain antibody fusion proteins

Single chain antibodies are produced by several methods including but not limited to: selection from phage libraries, cloning of the variable region of a specific antibody by cloning the cDNA of the antibody and using the flanking constant regions as the primer to clone the variable region, or by synthesizing an oligonucleotide corresponding to the variable region of any specific antibody. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The cell cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast.

In fusion molecules of the invention, the V_H and V_L can be linked by one of the following means or a combination thereof: a peptide linker between the C-terminus of the V_H and the N-terminus of the V_L; a Kex2p protease cleavage site between the V_H and V_L such that the two are cleaved apart upon secretion and then self associate; and cystine residues positioned such that the V_H and V_L can form a disulphide bond between them to link them together (see Figure 14). An alternative option would be to place the V_H at the N-terminus of HA or an HA domain fragment and the V_L at the C-terminus of the HA or HA domain fragment.

The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines. The antibody produced in this manner can be purified from media and tested for its binding to its antigen using standard immunochemical methods.

Example 9: Preparation of HA-cell adhesion molecule fusion proteins

The cDNA for the cell adhesion molecule of interest can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. . The

nucleotide sequences for the known cell adhesion molecules are known and available, for instance, in GenBank. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The cell adhesion molecule cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 10: Preparation of inhibitory factors and peptides as HA fusion proteins (such as HA-antiviral, HA-antibiotic, HA-enzyme inhibitor and HA-anti-allergic proteins)

The cDNA for the peptide of interest such as an antibiotic peptide can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The peptide cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 11: Preparation of targeted HA fusion proteins

The cDNA for the protein of interest can be isolated from cDNA library or can be made synthetically using several overlapping oligonucleotides using standard molecular

biology methods. The appropriate nucleotides can be engineered in the cDNA to form convenient restriction sites and also allow the attachment of the protein cDNA to albumin cDNA similar to the method described for hGH. Also a targeting protein or peptide cDNA such as single chain antibody or peptides, such as nuclear localization signals, that can direct proteins inside the cells can be fused to the other end of albumin. The protein of interest and the targeting peptide is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA which allows the fusion with albumin cDNA. In this manner both N- and C-terminal end of albumin are fused to other proteins. The fused cDNA is then excised from pPPC0005 and is inserted into a plasmid such as pSAC35 to allow the expression of the albumin fusion protein in yeast. All the above procedures can be performed using standard methods in molecular biology. The albumin fusion protein secreted from yeast can be collected and purified from the media and tested for its biological activity and its targeting activity using appropriate biochemical and biological tests.

Example 12: Preparation of HA-enzymes fusions

The cDNA for the enzyme of interest can be isolated by a variety of means including but not exclusively, from cDNA libraries, by RT-PCR and by PCR using a series of overlapping synthetic oligonucleotide primers, all using standard methods. The cDNA can be tailored at the 5' and 3' ends to generate restriction sites, such that oligonucleotide linkers can be used, for cloning of the cDNA into a vector containing the cDNA for HA. This can be at the N or C-terminus with or without the use of a spacer sequence. The enzyme cDNA is cloned into a vector such as pPPC0005 (Figure 4), pScCHSA, pScNHSA , or pC4:HSA from which the complete expression cassette is then excised and inserted into the plasmid pSAC35 to allow the expression of the albumin fusion protein in yeast. The albumin fusion protein secreted from the yeast can then be collected and purified from the media and tested for its biological activity. For expression in mammalian cell lines a similar procedure is adopted except that the expression cassette used employs a mammalian promoter, leader sequence and terminator (See Example 2). This expression cassette is then excised and inserted into a plasmid suitable for the transfection of mammalian cell lines.

Example 13: Bacterial Expression of an Albumin Fusion Protein

A polynucleotide encoding an albumin fusion protein of the present invention comprising a bacterial signal sequence is amplified using PCR oligonucleotide primers corresponding to the 5' and 3' ends of the DNA sequence, to synthesize insertion fragments. The primers used to amplify the polynucleotide encoding insert should preferably contain

restriction sites, such as BamHI and XbaI, at the 5' end of the primers in order to clone the amplified product into the expression vector. For example, BamHI and XbaI correspond to the restriction enzyme sites on the bacterial expression vector pQE-9. (Qiagen, Inc., Chatsworth, CA). This plasmid vector encodes antibiotic resistance (Amp^r), a bacterial origin of replication (ori), an IPTG-regulatable promoter/operator (P/O), a ribosome binding site (RBS), a 6-histidine tag (6-His), and restriction enzyme cloning sites.

The pQE-9 vector is digested with BamHI and XbaI and the amplified fragment is ligated into the pQE-9 vector maintaining the reading frame initiated at the bacterial RBS. The ligation mixture is then used to transform the E. coli strain M15/rep4 (Qiagen, Inc.) which contains multiple copies of the plasmid pREP4, which expresses the lacI repressor and also confers kanamycin resistance (Kan^r). Transformants are identified by their ability to grow on LB plates and ampicillin/kanamycin resistant colonies are selected. Plasmid DNA is isolated and confirmed by restriction analysis.

Clones containing the desired constructs are grown overnight (O/N) in liquid culture in LB media supplemented with both Amp (100 ug/ml) and Kan (25 ug/ml). The O/N culture is used to inoculate a large culture at a ratio of 1:100 to 1:250. The cells are grown to an optical density 600 ($O.D.^{600}$) of between 0.4 and 0.6. IPTG (Isopropyl-B-D-thiogalacto pyranoside) is then added to a final concentration of 1 mM. IPTG induces by inactivating the lacI repressor, clearing the P/O leading to increased gene expression.

Cells are grown for an extra 3 to 4 hours. Cells are then harvested by centrifugation (20 mins at 6000Xg). The cell pellet is solubilized in the chaotropic agent 6 Molar Guanidine HCl or preferably in 8 M urea and concentrations greater than 0.14 M 2-mercaptoethanol by stirring for 3-4 hours at 4°C (see, e.g., Burton et al., Eur. J. Biochem. *179*:379-387 (1989)). The cell debris is removed by centrifugation, and the supernatant containing the polypeptide is loaded onto a nickel-nitrilo-tri-acetic acid ("Ni-NTA") affinity resin column (available from QIAGEN, Inc., *supra*). Proteins with a 6 x His tag bind to the Ni-NTA resin with high affinity and can be purified in a simple one-step procedure (for details see: The QIAexpressionist (1995) QIAGEN, Inc., *supra*).

Briefly, the supernatant is loaded onto the column in 6 M guanidine-HCl, pH 8. The column is first washed with 10 volumes of 6 M guanidine-HCl, pH 8, then washed with 10 volumes of 6 M guanidine-HCl pH 6, and finally the polypeptide is eluted with 6 M guanidine-HCl, pH 5.

The purified protein is then renatured by dialyzing it against phosphate-buffered saline (PBS) or 50 mM Na-acetate, pH 6 buffer plus 200 mM NaCl. Alternatively, the protein can be successfully refolded while immobilized on the Ni-NTA column. Exemplary conditions are as follows: renature using a linear 6M-1M urea gradient in 500 mM NaCl, 20% glycerol, 20

mM Tris/HCl pH 7.4, containing protease inhibitors. The renaturation should be performed over a period of 1.5 hours or more. After renaturation the proteins are eluted by the addition of 250 mM immidazole. Immidazole is removed by a final dialyzing step against PBS or 50 mM sodium acetate pH 6 buffer plus 200 mM NaCl. The purified protein is stored at 4° C or frozen at -80° C.

In addition to the above expression vector, the present invention further includes an expression vector, called pHE4a (ATCC Accession Number 209645, deposited on February 25, 1998) which contains phage operator and promoter elements operatively linked to a polynucleotide encoding an albumin fusion protein of the present invention, called pHE4a. (ATCC Accession Number 209645, deposited on February 25, 1998.) This vector contains: 1) a neomycinphosphotransferase gene as a selection marker, 2) an E. coli origin of replication, 3) a T5 phage promoter sequence, 4) two lac operator sequences, 5) a Shine-Delgarno sequence, and 6) the lactose operon repressor gene (lacIq). The origin of replication (oriC) is derived from pUC19 (LTI, Gaithersburg, MD). The promoter and operator sequences are made synthetically.

DNA can be inserted into the pHE4a by restricting the vector with NdeI and XbaI, BamHI, XhoI, or Asp718, running the restricted product on a gel, and isolating the larger fragment (the stuffer fragment should be about 310 base pairs). The DNA insert is generated according to PCR protocols described herein or otherwise known in the art, using PCR primers having restriction sites for NdeI (5' primer) and XbaI, BamHI, XhoI, or Asp718 (3' primer). The PCR insert is gel purified and restricted with compatible enzymes. The insert and vector are ligated according to standard protocols.

The engineered vector may be substituted in the above protocol to express protein in a bacterial system.

Example 14: Expression of an Albumin Fusion Protein in Mammalian Cells

The albumin fusion proteins of the present invention can be expressed in a mammalian cell. A typical mammalian expression vector contains a promoter element, which mediates the initiation of transcription of mRNA, a protein coding sequence, and signals required for the termination of transcription and polyadenylation of the transcript. Additional elements include enhancers, Kozak sequences and intervening sequences flanked by donor and acceptor sites for RNA splicing. Highly efficient transcription is achieved with the early and late promoters from SV40, the long terminal repeats (LTRs) from Retroviruses, e.g., RSV, HTLVI, HIVI and the early promoter of the cytomegalovirus (CMV). However, cellular elements can also be used (e.g., the human actin promoter).

Suitable expression vectors for use in practicing the present invention include, for example, vectors such as, pSVL and pMSG (Pharmacia, Uppsala, Sweden), pRSVcat (ATCC 37152), pSV2dhfr (ATCC 37146), pBC12MI (ATCC 67109), pCMVSport 2.0, and pCMVSport 3.0. Mammalian host cells that could be used include, but are not limited to, human Hela, 293, H9 and Jurkat cells, mouse NIH3T3 and C127 cells, Cos 1, Cos 7 and CV1, quail QC1-3 cells, mouse L cells and Chinese hamster ovary (CHO) cells.

Alternatively, the albumin fusion protein can be expressed in stable cell lines containing the polynucleotide encoding the albumin fusion protein integrated into a chromosome. The co-transfection with a selectable marker such as DHFR, gpt, neomycin, or hygromycin allows the identification and isolation of the transfected cells.

The transfected polynucleotide encoding the fusion protein can also be amplified to express large amounts of the encoded fusion protein. The DHFR (dihydrofolate reductase) marker is useful in developing cell lines that carry several hundred or even several thousand copies of the gene of interest. (See, e.g., Alt et al., J. Biol. Chem. 253:1357-1370 (1978); Hamlin et al., Biochem. et Biophys. Acta, 1097:107-143 (1990); Page et al., Biotechnology 9:64-68 (1991)). Another useful selection marker is the enzyme glutamine synthase (GS) (Murphy et al., Biochem J. 227:277-279 (1991); Bebbington et al., Bio/Technology 10:169-175 (1992). Using these markers, the mammalian cells are grown in selective medium and the cells with the highest resistance are selected. These cell lines contain the amplified gene(s) integrated into a chromosome. Chinese hamster ovary (CHO) and NSO cells are often used for the production of proteins.

Derivatives of the plasmid pSV2-dhfr (ATCC Accession No. 37146), the expression vectors pC4 (ATCC Accession No. 209646) and pC6 (ATCC Accession No.209647) contain the strong promoter (LTR) of the Rous Sarcoma Virus (Cullen et al., Molecular and Cellular Biology, 438-447 (March, 1985)) plus a fragment of the CMV-enhancer (Boshart et al., Cell 41:521-530 (1985)). Multiple cloning sites, e.g., with the restriction enzyme cleavage sites BamHI, XbaI and Asp718, facilitate the cloning of the gene of interest. The vectors also contain the 3' intron, the polyadenylation and termination signal of the rat preproinsulin gene, and the mouse DHFR gene under control of the SV40 early promoter.

Specifically, the plasmid pC6, for example, is digested with appropriate restriction enzymes and then dephosphorylated using calf intestinal phosphates by procedures known in the art. The vector is then isolated from a 1% agarose gel.

A polynucleotide encoding an albumin fusion protein of the present invention is generated using techniques known in the art and this polynucleotide is amplified using PCR technology known in the art. If a naturally occurring signal sequence is used to produce the fusion protein of the present invention, the vector does not need a second signal peptide.

Alternatively, if a naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International Publication No. WO 96/34891.)

The amplified fragment encoding the fusion protein of the invention is isolated from a 1% agarose gel using a commercially available kit ("Geneclean," BIO 101 Inc., La Jolla, Ca.). The fragment then is digested with appropriate restriction enzymes and again purified on a 1% agarose gel.

The amplified fragment encoding the albumin fusion protein of the invention is then digested with the same restriction enzyme and purified on a 1% agarose gel. The isolated fragment and the dephosphorylated vector are then ligated with T4 DNA ligase. *E. coli* HB101 or XL-1 Blue cells are then transformed and bacteria are identified that contain the fragment inserted into plasmid pC6 using, for instance, restriction enzyme analysis.

Chinese hamster ovary cells lacking an active DHFR gene is used for transfection. Five μg of the expression plasmid pC6 or pC4 is cotransfected with 0.5 μg of the plasmid pSVneo using lipofectin (Felgner et al., *supra*). The plasmid pSV2-neo contains a dominant selectable marker, the *neo* gene from Tn5 encoding an enzyme that confers resistance to a group of antibiotics including G418. The cells are seeded in alpha minus MEM supplemented with 1 mg/ml G418. After 2 days, the cells are trypsinized and seeded in hybridoma cloning plates (Greiner, Germany) in alpha minus MEM supplemented with 10, 25, or 50 ng/ml of methotrexate plus 1 mg/ml G418. After about 10-14 days single clones are trypsinized and then seeded in 6-well petri dishes or 10 ml flasks using different concentrations of methotrexate (50 nM, 100 nM, 200 nM, 400 nM, 800 nM). Clones growing at the highest concentrations of methotrexate are then transferred to new 6-well plates containing even higher concentrations of methotrexate (1 μM, 2 μM, 5 μM, 10 mM, 20 mM). The same procedure is repeated until clones are obtained which grow at a concentration of 100 - 200 μM. Expression of the desired fusion protein is analyzed, for instance, by SDS-PAGE and Western blot or by reversed phase HPLC analysis.

Example 15: Multifusion Fusions

The albumin fusion proteins (e.g,. containing a Therapeutic protein (or fragment or variant thereof) fused to albumin (or a fragment or variant thereof)) may additionally be fused to other proteins to generate "multifusion proteins". These multifusion proteins can be used for a variety of applications. For example, fusion of the albumin fusion proteins of the invention to His-tag, HA-tag, protein A, IgG domains, and maltose binding protein facilitates purification. (See e.g,. EP A 394,827; Traunecker et al., Nature 331:84-86 (1988)). Nuclear localization signals fused to the polypeptides of the present invention can target the protein to

a specific subcellular localization, while covalent heterodimer or homodimers can increase or decrease the activity of an albumin fusion protein. Furthermore, the fusion of additional protein sequences to the albumin fusion proteins of the invention may further increase the solubility and/or stability of the fusion protein. The fusion proteins described above can be made using or routinely modifting techniques known in the art and/or by modifying the following protocol, which outlines the fusion of a polypeptide to an IgG molecule.

Briefly, the human Fc portion of the IgG molecule can be PCR amplified, using primers that span the 5' and 3' ends of the sequence described below. These primers also should have convenient restriction enzyme sites that will facilitate cloning into an expression vector, preferably a mammalian or yeast expression vector.

For example, if pC4 (ATCC Accession No. 209646) is used, the human Fc portion can be ligated into the BamHI cloning site. Note that the 3' BamHI site should be destroyed. Next, the vector containing the human Fc portion is re-restricted with BamHI, linearizing the vector, and a polynucleotide encoding an albumin fusion protein of the present invention (generateed and isolated using techniques known in the art), is ligated into this BamHI site. Note that the polynucleotide encoding the fusion protein of the invention is cloned without a stop codon, otherwise a Fc containing fusion protein will not be produced.

If the naturally occurring signal sequence is used to produce the albumin fusion protein of the present invention, pC4 does not need a second signal peptide. Alternatively, if the naturally occurring signal sequence is not used, the vector can be modified to include a heterologous signal sequence. (See, e.g., International Publication No. WO 96/34891.)

Human IgG Fc region:

GGGATCCGGAGCCCAAATCTTCTGACAAAACTCACACATGCCCACCGTGC
CCAGCACCTGAATTCGAGGGTGCACCGTCAGTCTTCCTCTTCCCCCCAAAACCCA
AGGACACCCTCATGATCTCCCGGACTCCTGAGGTCACATGCGTGGTGGTGGACG
TAAGCCACGAAGACCCTGAGGTCAAGTTCAACTGGTACGTGGACGGCGTGGAGG
TGCATAATGCCAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTG
TGGTCAGCGTCCTCACCGTCCTGCACCAGGACTGGCTGAATGGCAAGGAGTACA
AGTGCAAGGTCTCCAACAAAGCCCTCCCAACCCCCATCGAGAAAACCATCTCCAA
AGCCAAAGGGCAGCCCCGAGAACCACAGGTGTACACCCTGCCCCCATCCCGGGA
TGAGCTGACCAAGAACCAGGTCAGCCTGACCTGCCTGGTCAAAGGCTTCTATCCA
AGCGACATCGCCGTGGAGTGGGAGAGCAATGGGCAGCCGGAGAACAACTACAA
GACCACGCCTCCCGTGCTGGACTCCGACGGCTCCTTCTTCCTCTACAGCAAGCTC
ACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATG
CATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCTCCGGGTA

AATGAGTGCGACGGCCGCGACTCTAGAGGAT (SEQ ID NO: 36)

Example 16: Production of an Antibody from an Albumin Fusion Protein

a) Hybridoma Technology

Antibodies that bind the albumin fusion proteins of the present invention and portions of the albumin fusion proteins of the present invention (e.g., the Therapeutic protein portion or albumin portion of the fusion protein) can be prepared by a variety of methods. (See, Current Protocols, Chapter 2.) As one example of such methods, a preparation of an albumin fusion protein of the invention or a portion of an albumin fusion protein of the invention is prepared and purified to render it substantially free of natural contaminants. Such a preparation is then introduced into an animal in order to produce polyclonal antisera of greater specific activity.

Monoclonal antibodies specific for an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, are prepared using hybridoma technology (Kohler et al., Nature 256:495 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976); Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling et al., in: Monoclonal Antibodies and T-Cell Hybridomas, Elsevier, N.Y., pp. 563-681 (1981)). In general, an animal (preferably a mouse) is immunized with an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention. The splenocytes of such mice are extracted and fused with a suitable myeloma cell line. Any suitable myeloma cell line may be employed in accordance with the present invention; however, it is preferable to employ the parent myeloma cell line (SP2O), available from the ATCC. After fusion, the resulting hybridoma cells are selectively maintained in HAT medium, and then cloned by limiting dilution as described by Wands et al. (Gastroenterology 80:225-232 (1981)). The hybridoma cells obtained through such a selection are then assayed to identify clones which secrete antibodies capable of binding an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention.

Alternatively, additional antibodies capable of binding to an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention can be produced in a two-step procedure using anti-idiotypic antibodies. Such a method makes use of the fact that antibodies are themselves antigens, and therefore, it is possible to obtain an antibody which binds to a second antibody. In accordance with this method, protein specific antibodies are used to immunize an animal, preferably a mouse. The splenocytes of such an animal are then used to produce hybridoma cells, and the hybridoma cells are screened to identify clones which produce an antibody whose ability to bind to the an albumin fusion protein of the

invention (or portion of an albumin fusion protein of the invention) -specific antibody can be blocked by the fusion protein of the invention, or a portion of an albumin fusion protein of the invention. Such antibodies comprise anti-idiotypic antibodies to the fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibody and are used to immunize an animal to induce formation of further fusion protein of the invention (or portion of an albumin fusion protein of the invention) -specific antibodies.

For *in vivo* use of antibodies in humans, an antibody is "humanized". Such antibodies can be produced using genetic constructs derived from hybridoma cells producing the monoclonal antibodies described above. Methods for producing chimeric and humanized antibodies are known in the art and are discussed herein. (See, for review, Morrison, Science 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986); Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et al., EP 171496; Morrison et al., EP 173494; Neuberger et al., WO 8601533; Robinson et al., International Publication No. WO 8702671; Boulianne et al., Nature 312:643 (1984); Neuberger et al., Nature 314:268 (1985)).

b) Isolation Of Antibody Fragments Directed Against an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention From A Library Of scFvs

Naturally occurring V-genes isolated from human PBLs are constructed into a library of antibody fragments which contain reactivities against an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, to which the donor may or may not have been exposed (see e.g., U.S. Patent 5,885,793 incorporated herein by reference in its entirety).

Rescue of the Library. A library of scFvs is constructed from the RNA of human PBLs as described in International Publication No. WO 92/01047. To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100 μg/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture is used to inoculate 50 ml of 2xTY-AMP-GLU, 2 x 108 TU of delta gene 3 helper (M13 delta gene III, see International Publication No. WO 92/01047) are added and the culture incubated at 37°C for 45 minutes without shaking and then at 37°C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 μg/ml ampicillin and 50 ug/ml kanamycin and grown overnight. Phage are prepared as described in International Publication No. WO 92/01047.

M13 delta gene III is prepared as follows: M13 delta gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a greater

avidity of binding to antigen. Infectious M13 delta gene III particles are made by growing the helper phage in cells harboring a pUC19 derivative supplying the wild type gene III protein during phage morphogenesis. The culture is incubated for 1 hour at 37° C without shaking and then for a further hour at 37°C with shaking. Cells are spun down (IEC-Centra 8,400 r.p.m. for 10 min), resuspended in 300 ml 2xTY broth containing 100 μg ampicillin/ml and 25 μg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking at 37°C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations (Sambrook et al., 1990), resuspended in 2 ml PBS and passed through a 0.45 μm filter (Minisart NML; Sartorius) to give a final concentration of approximately 10^{13} transducing units/ml (ampicillin-resistant clones).

Panning of the Library. Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 μg/ml or 10 μg/ml of an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention. Tubes are blocked with 2% Marvel-PBS for 2 hours at 37°C and then washed 3 times in PBS. Approximately 10^{13} TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log E. coli TG1 by incubating eluted phage with bacteria for 30 minutes at 37°C. The E. coli are then plated on TYE plates containing 1% glucose and 100 μg/ml ampicillin. The resulting bacterial library is then rescued with delta gene 3 helper phage as described above to prepare phage for a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Characterization of Binders. Eluted phage from the 3rd and 4th rounds of selection are used to infect E. coli HB 2151 and soluble scFv is produced (Marks, et al., 1991) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of an albumin fusion protein of the invention, or a portion of an albumin fusion protein of the invention, in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., International Publication No. WO 92/01047) and then by sequencing. These ELISA positive clones may also be further characterized by techniques known in the art, such as, for example, epitope mapping, binding affinity, receptor signal transduction, ability to block or competitively inhibit antibody/antigen binding, and competitive agonistic or antagonistic activity.

Example 17: Method of Treatment Using Gene Therapy-Ex Vivo

One method of gene therapy transplants fibroblasts, which are capable of expressing an albumin fusion protein of the present invention, onto a patient. Generally, fibroblasts are obtained from a subject by skin biopsy. The resulting tissue is placed in tissue-culture medium and separated into small pieces. Small chunks of the tissue are placed on a wet surface of a tissue culture flask, approximately ten pieces are placed in each flask. The flask is turned upside down, closed tight and left at room temperature over night. After 24 hours at room temperature, the flask is inverted and the chunks of tissue remain fixed to the bottom of the flask and fresh media (e.g., Ham's F12 media, with 10% FBS, penicillin and streptomycin) is added. The flasks are then incubated at 37 degree C for approximately one week.

At this time, fresh media is added and subsequently changed every several days. After an additional two weeks in culture, a monolayer of fibroblasts emerge. The monolayer is trypsinized and scaled into larger flasks.

pMV-7 (Kirschmeier, P.T. et al., DNA, 7:219-25 (1988)), flanked by the long terminal repeats of the Moloney murine sarcoma virus, is digested with EcoRI and HindIII and subsequently treated with calf intestinal phosphatase. The linear vector is fractionated on agarose gel and purified, using glass beads.

Polynucleotides encoding an albumin fusion protein of the invention can be generated using techniques known in the art amplified using PCR primers which correspond to the 5' and 3' end sequences and optionally having appropriate restriction sites and initiation/stop codons, if necessary. Preferably, the 5' primer contains an EcoRI site and the 3' primer includes a HindIII site. Equal quantities of the Moloney murine sarcoma virus linear backbone and the amplified EcoRI and HindIII fragment are added together, in the presence of T4 DNA ligase. The resulting mixture is maintained under conditions appropriate for ligation of the two fragments. The ligation mixture is then used to transform bacteria HB101, which are then plated onto agar containing kanamycin for the purpose of confirming that the vector has the gene of interest properly inserted.

The amphotropic pA317 or GP+am12 packaging cells are grown in tissue culture to confluent density in Dulbecco's Modified Eagles Medium (DMEM) with 10% calf serum (CS), penicillin and streptomycin. The MSV vector containing the gene is then added to the media and the packaging cells transduced with the vector. The packaging cells now produce infectious viral particles containing the gene (the packaging cells are now referred to as producer cells).

Fresh media is added to the transduced producer cells, and subsequently, the media is

harvested from a 10 cm plate of confluent producer cells. The spent media, containing the infectious viral particles, is filtered through a millipore filter to remove detached producer cells and this media is then used to infect fibroblast cells. Media is removed from a sub-confluent plate of fibroblasts and quickly replaced with the media from the producer cells. This media is removed and replaced with fresh media. If the titer of virus is high, then virtually all fibroblasts will be infected and no selection is required. If the titer is very low, then it is necessary to use a retroviral vector that has a selectable marker, such as neo or his. Once the fibroblasts have been efficiently infected, the fibroblasts are analyzed to determine whether the albumin fusion protein is produced.

The engineered fibroblasts are then transplanted onto the host, either alone or after having been grown to confluence on cytodex 3 microcarrier beads.

Example 18: Method of Treatment Using Gene Therapy - In Vivo

Another aspect of the present invention is using *in vivo* gene therapy methods to treat disorders, diseases and conditions. The gene therapy method relates to the introduction of naked nucleic acid (DNA, RNA, and antisense DNA or RNA) sequences encoding an albumin fusion protein of the invention into an animal. Polynucleotides encoding albumin fusion proteins of the present invention may be operatively linked to (i.e., associated with) a promoter or any other genetic elements necessary for the expression of the polypeptide by the target tissue. Such gene therapy and delivery techniques and methods are known in the art, see, for example, WO90/11092, WO98/11779; U.S. Patent NO. 5693622, 5705151, 5580859; Tabata et al., Cardiovasc. Res. 35(3):470-479 (1997); Chao et al., Pharmacol. Res. 35(6):517-522 (1997); Wolff, Neuromuscul. Disord. 7(5):314-318 (1997); Schwartz et al., Gene Ther. 3(5):405-411 (1996); Tsurumi et al., Circulation 94(12):3281-3290 (1996) (incorporated herein by reference).

The polynucleotide constructs may be delivered by any method that delivers injectable materials to the cells of an animal, such as, injection into the interstitial space of tissues (heart, muscle, skin, lung, liver, intestine and the like). The polynucleotide constructs can be delivered in a pharmaceutically acceptable liquid or aqueous carrier.

The term "naked" polynucleotide, DNA or RNA, refers to sequences that are free from any delivery vehicle that acts to assist, promote, or facilitate entry into the cell, including viral sequences, viral particles, liposome formulations, lipofectin or precipitating agents and the like. However, polynucleotides encoding albumin fusion proteins of the present invention may also be delivered in liposome formulations (such as those taught in Felgner P.L. et al. (1995) Ann. NY Acad. Sci. 772:126-139 and Abdallah B. et al. (1995) Biol. Cell 85(1):1-7) which can be prepared by methods well known to those skilled in the art.

The polynucleotide vector constructs used in the gene therapy method are preferably constructs that will not integrate into the host genome nor will they contain sequences that allow for replication. Any strong promoter known to those skilled in the art can be used for driving the expression of DNA. Unlike other gene therapy techniques, one major advantage of introducing naked nucleic acid sequences into target cells is the transitory nature of the polynucleotide synthesis in the cells. Studies have shown that non-replicating DNA sequences can be introduced into cells to provide production of the desired polypeptide for periods of up to six months.

The polynucleotide construct can be delivered to the interstitial space of tissues within an animal, including muscle, skin, brain, lung, liver, spleen, bone marrow, thymus, heart, lymph, blood, bone, cartilage, pancreas, kidney, gall bladder, stomach, intestine, testis, ovary, uterus, rectum, nervous system, eye, gland, and connective tissue. Interstitial space of the tissues comprises the intercellular fluid, mucopolysaccharide matrix among the reticular fibers of organ tissues, elastic fibers in the walls of vessels or chambers, collagen fibers of fibrous tissues, or that same matrix within connective tissue ensheathing muscle cells or in the lacunae of bone. It is similarly the space occupied by the plasma of the circulation and the lymph fluid of the lymphatic channels. Delivery to the interstitial space of muscle tissue is preferred for the reasons discussed below. They may be conveniently delivered by injection into the tissues comprising these cells. They are preferably delivered to and expressed in persistent, non-dividing cells which are differentiated, although delivery and expression may be achieved in non-differentiated or less completely differentiated cells, such as, for example, stem cells of blood or skin fibroblasts. *In vivo* muscle cells are particularly competent in their ability to take up and express polynucleotides.

For the naked polynucleotide injection, an effective dosage amount of DNA or RNA will be in the range of from about 0.05 g/kg body weight to about 50 mg/kg body weight. Preferably the dosage will be from about 0.005 mg/kg to about 20 mg/kg and more preferably from about 0.05 mg/kg to about 5 mg/kg. Of course, as the artisan of ordinary skill will appreciate, this dosage will vary according to the tissue site of injection. The appropriate and effective dosage of nucleic acid sequence can readily be determined by those of ordinary skill in the art and may depend on the condition being treated and the route of administration. The preferred route of administration is by the parenteral route of injection into the interstitial space of tissues. However, other parenteral routes may also be used, such as, inhalation of an aerosol formulation particularly for delivery to lungs or bronchial tissues, throat or mucous membranes of the nose. In addition, naked polynucleotide constructs can be delivered to arteries during angioplasty by the catheter used in the procedure.

The dose response effects of injected polynucleotide in muscle *in vivo* is determined

as follows. Suitable template DNA for production of mRNA coding for polypeptide of the present invention is prepared in accordance with a standard recombinant DNA methodology. The template DNA, which may be either circular or linear, is either used as naked DNA or complexed with liposomes. The quadriceps muscles of mice are then injected with various amounts of the template DNA.

Five to six week old female and male Balb/C mice are anesthetized by intraperitoneal injection with 0.3 ml of 2.5% Avertin. A 1.5 cm incision is made on the anterior thigh, and the quadriceps muscle is directly visualized. The template DNA is injected in 0.1 ml of carrier in a 1 cc syringe through a 27 gauge needle over one minute, approximately 0.5 cm from the distal insertion site of the muscle into the knee and about 0.2 cm deep. A suture is placed over the injection site for future localization, and the skin is closed with stainless steel clips.

After an appropriate incubation time (e.g., 7 days) muscle extracts are prepared by excising the entire quadriceps. Every fifth 15 um cross-section of the individual quadriceps muscles is histochemically stained for protein expression. A time course for fusion protein expression may be done in a similar fashion except that quadriceps from different mice are harvested at different times. Persistence of DNA in muscle following injection may be determined by Southern blot analysis after preparing total cellular DNA and HIRT supernatants from injected and control mice. The results of the above experimentation in mice can be used to extrapolate proper dosages and other treatment parameters in humans and other animals using naked DNA.

Example 19: Transgenic Animals

The albumin fusion proteins of the invention can also be expressed in transgenic animals. Animals of any species, including, but not limited to, mice, rats, rabbits, hamsters, guinea pigs, pigs, micro-pigs, goats, sheep, cows and non-human primates, *e.g.*, baboons, monkeys, and chimpanzees may be used to generate transgenic animals. In a specific embodiment, techniques described herein or otherwise known in the art, are used to express fusion proteins of the invention in humans, as part of a gene therapy protocol.

Any technique known in the art may be used to introduce the polynucleotides encoding the albumin fusion proteins of the invention into animals to produce the founder lines of transgenic animals. Such techniques include, but are not limited to, pronuclear microinjection (Paterson et al., Appl. Microbiol. Biotechnol. 40:691-698 (1994); Carver et al., Biotechnology (NY) 11:1263-1270 (1993); Wright et al., Biotechnology (NY) 9:830-834 (1991); and Hoppe et al., U.S. Pat. No. 4,873,191 (1989)); retrovirus mediated gene transfer into germ lines (Van der Putten et al., Proc. Natl. Acad. Sci., USA 82:6148-6152 (1985)), blastocysts or embryos; gene targeting in embryonic stem cells (Thompson et al., Cell

56:313-321 (1989)); electroporation of cells or embryos (Lo, 1983, Mol Cell. Biol. 3:1803-1814 (1983)); introduction of the polynucleotides of the invention using a gene gun (see, e.g., Ulmer et al., Science 259:1745 (1993); introducing nucleic acid constructs into embryonic pleuripotent stem cells and transferring the stem cells back into the blastocyst; and sperm-mediated gene transfer (Lavitrano et al., Cell 57:717-723 (1989); etc. For a review of such techniques, see Gordon, "Transgenic Animals," Intl. Rev. Cytol. 115:171-229 (1989), which is incorporated by reference herein in its entirety.

Any technique known in the art may be used to produce transgenic clones containing polynucleotides encoding albumin fusion proteins of the invention, for example, nuclear transfer into enucleated oocytes of nuclei from cultured embryonic, fetal, or adult cells induced to quiescence (Campell et al., Nature 380:64-66 (1996); Wilmut et al., Nature 385:810-813 (1997)).

The present invention provides for transgenic animals that carry the polynucleotides encoding the albumin fusion proteins of the invention in all their cells, as well as animals which carry these polynucleotides in some, but not all their cells, *i.e.*, mosaic animals or chimeric. The transgene may be integrated as a single transgene or as multiple copies such as in concatamers, *e.g.*, head-to-head tandems or head-to-tail tandems. The transgene may also be selectively introduced into and activated in a particular cell type by following, for example, the teaching of Lasko et al. (Lasko et al., Proc. Natl. Acad. Sci. USA 89:6232-6236 (1992)). The regulatory sequences required for such a cell-type specific activation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art. When it is desired that the polynucleotide encoding the fusion protein of the invention be integrated into the chromosomal site of the endogenous gene corresponding to the Therapeutic protein portion or ablumin portion of the fusion protein of the invention, gene targeting is preferred. Briefly, when such a technique is to be utilized, vectors containing some nucleotide sequences homologous to the endogenous gene are designed for the purpose of integrating, via homologous recombination with chromosomal sequences, into and disrupting the function of the nucleotide sequence of the endogenous gene. The transgene may also be selectively introduced into a particular cell type, thus inactivating the endogenous gene in only that cell type, by following, for example, the teaching of Gu et al. (Gu et al., Science 265:103-106 (1994)). The regulatory sequences required for such a cell-type specific inactivation will depend upon the particular cell type of interest, and will be apparent to those of skill in the art.

Once transgenic animals have been generated, the expression of the recombinant gene may be assayed utilizing standard techniques. Initial screening may be accomplished by Southern blot analysis or PCR techniques to analyze animal tissues to verify that integration of the polynucleotide encoding the fsuion protien of the invention has taken place. The level

of mRNA expression of the polynucleotide encoding the fusion protein of the invention in the tissues of the transgenic animals may also be assessed using techniques which include, but are not limited to, Northern blot analysis of tissue samples obtained from the animal, *in situ* hybridization analysis, and reverse transcriptase-PCR (rt-PCR). Samples of fusion protein-expressing tissue may also be evaluated immunocytochemically or immunohistochemically using antibodies specific for the fusion protein.

Once the founder animals are produced, they may be bred, inbred, outbred, or crossbred to produce colonies of the particular animal. Examples of such breeding strategies include, but are not limited to: outbreeding of founder animals with more than one integration site in order to establish separate lines; inbreeding of separate lines in order to produce compound transgenics that express the transgene at higher levels because of the effects of additive expression of each transgene; crossing of heterozygous transgenic animals to produce animals homozygous for a given integration site in order to both augment expression and eliminate the need for screening of animals by DNA analysis; crossing of separate homozygous lines to produce compound heterozygous or homozygous lines; and breeding to place the transgene (i.e., polynucleotide encoding an albumin fusion protein of the invention) on a distinct background that is appropriate for an experimental model of interest.

Transgenic animals of the invention have uses which include, but are not limited to, animal model systems useful in elaborating the biological function of fusion proteins of the invention and the Therapeutic protein and/or albumin component of the fusion protein of the invention, studying conditions and/or disorders associated with aberrant expression, and in screening for compounds effective in ameliorating such conditions and/or disorders.

Example 20: Assays Detecting Stimulation or Inhibition of B cell Proliferation and Differentiation

Generation of functional humoral immune responses requires both soluble and cognate signaling between B-lineage cells and their microenvironment. Signals may impart a positive stimulus that allows a B-lineage cell to continue its programmed development, or a negative stimulus that instructs the cell to arrest its current developmental pathway. To date, numerous stimulatory and inhibitory signals have been found to influence B cell responsiveness including IL-2, IL-4, IL-5, IL-6, IL-7, IL10, IL-13, IL-14 and IL-15. Interestingly, these signals are by themselves weak effectors but can, in combination with various co-stimulatory proteins, induce activation, proliferation, differentiation, homing, tolerance and death among B cell populations.

One of the best studied classes of B-cell co-stimulatory proteins is the TNF-superfamily. Within this family CD40, CD27, and CD30 along with their respective ligands

CD154, CD70, and CD153 have been found to regulate a variety of immune responses. Assays which allow for the detection and/or observation of the proliferation and differentiation of these B-cell populations and their precursors are valuable tools in determining the effects various proteins may have on these B-cell populations in terms of proliferation and differentiation. Listed below are two assays designed to allow for the detection of the differentiation, proliferation, or inhibition of B-cell populations and their precursors.

In Vitro Assay- Albumin fusion proteins of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin) can be assessed for its ability to induce activation, proliferation, differentiation or inhibition and/or death in B-cell populations and their precursors. The activity of an albumin fusion protein of the invention on purified human tonsillar B cells, measured qualitatively over the dose range from 0.1 to 10,000 ng/mL, is assessed in a standard B-lymphocyte co-stimulation assay in which purified tonsillar B cells are cultured in the presence of either formalin-fixed *Staphylococcus aureus* Cowan I (SAC) or immobilized anti-human IgM antibody as the priming agent. Second signals such as IL-2 and IL-15 synergize with SAC and IgM crosslinking to elicit B cell proliferation as measured by tritiated-thymidine incorporation. Novel synergizing agents can be readily identified using this assay. The assay involves isolating human tonsillar B cells by magnetic bead (MACS) depletion of CD3-positive cells. The resulting cell population is greater than 95% B cells as assessed by expression of CD45R(B220).

Various dilutions of each sample are placed into individual wells of a 96-well plate to which are added 10^5 B-cells suspended in culture medium (RPMI 1640 containing 10% FBS, 5 X 10^{-5}M 2ME, 100U/ml penicillin, 10ug/ml streptomycin, and 10^{-5} dilution of SAC) in a total volume of 150ul. Proliferation or inhibition is quantitated by a 20h pulse (1uCi/well) with 3H-thymidine (6.7 Ci/mM) beginning 72h post factor addition. The positive and negative controls are IL2 and medium respectively.

In vivo Assay- BALB/c mice are injected (i.p.) twice per day with buffer only, or 2 mg/Kg of an albumin fusion protein of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin). Mice receive this treatment for 4 consecutive days, at which time they are sacrificed and various tissues and serum collected for analyses. Comparison of H&E sections from normal spleens and spleens treated with the albumin fusion protein of the invention identify the results of the activity of the fusion protein on spleen cells, such as the diffusion of peri-arterial lymphatic sheaths, and/or significant increases in the nucleated cellularity of the red pulp regions, which may indicate the activation of the differentiation and proliferation of B-

cell populations. Immunohistochemical studies using a B cell marker, anti-CD45R(B220), are used to determine whether any physiological changes to splenic cells, such as splenic disorganization, are due to increased B-cell representation within loosely defined B-cell zones that infiltrate established T-cell regions.

Flow cytometric analyses of the spleens from mice treated with the albumin fusion protein is used to indicate whether the albumin fusion protein specifically increases the proportion of ThB+, CD45R(B220)dull B cells over that which is observed in control mice.

Likewise, a predicted consequence of increased mature B-cell representation *in vivo* is a relative increase in serum Ig titers. Accordingly, serum IgM and IgA levels are compared between buffer and fusion protein treated mice.

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 21: T Cell Proliferation Assay

A CD3-induced proliferation assay is performed on PBMCs and is measured by the uptake of ^{3}H-thymidine. The assay is performed as follows. Ninety-six well plates are coated with 100 µl/well of mAb to CD3 (HIT3a, Pharmingen) or isotype-matched control mAb (B33.1) overnight at 4 degrees C (1 µg/ml in .05M bicarbonate buffer, pH 9.5), then washed three times with PBS. PBMC are isolated by F/H gradient centrifugation from human peripheral blood and added to quadruplicate wells (5 x 10^{4}/well) of mAb coated plates in RPMI containing 10% FCS and P/S in the presence of varying concentrations of an albumin fusion protein of the invention (including fusion proteins containing fragments or variants of Therapeutic proteins and/or albumin or fragments or variants of albumin) (total volume 200 ul). Relevant protein buffer and medium alone are controls. After 48 hr. culture at 37 degrees C, plates are spun for 2 min. at 1000 rpm and 100 µl of supernatant is removed and stored –20 degrees C for measurement of IL-2 (or other cytokines) if effect on proliferation is observed. Wells are supplemented with 100 ul of medium containing 0.5 uCi of ^{3}H-thymidine and cultured at 37 degrees C for 18-24 hr. Wells are harvested and incorporation of ^{3}H-thymidine used as a measure of proliferation. Anti-CD3 alone is the positive control for proliferation. IL-2 (100 U/ml) is also used as a control which enhances proliferation. Control antibody which does not induce proliferation of T cells is used as the negative control for the effects of fusion proteins of the invention.

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test

the activity of fusion proteins or polynucleotides of the invention (e.g., gene therapy).

Example 22: Effect of Fusion Proteins of the Invention on the Expression of MHC Class II, Costimulatory and Adhesion Molecules and Cell Differentiation of Monocytes and Monocyte-Derived Human Dendritic Cells

Dendritic cells are generated by the expansion of proliferating precursors found in the peripheral blood: adherent PBMC or elutriated monocytic fractions are cultured for 7-10 days with GM-CSF (50 ng/ml) and IL-4 (20 ng/ml). These dendritic cells have the characteristic phenotype of immature cells (expression of CD1, CD80, CD86, CD40 and MHC class II antigens). Treatment with activating factors, such as TNF-α, causes a rapid change in surface phenotype (increased expression of MHC class I and II, costimulatory and adhesion molecules, downregulation of FCγRII, upregulation of CD83). These changes correlate with increased antigen-presenting capacity and with functional maturation of the dendritic cells.

FACS analysis of surface antigens is performed as follows. Cells are treated 1-3 days with increasing concentrations of an albumin fusion protein of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Effect on the production of cytokines. Cytokines generated by dendritic cells, in particular IL-12, are important in the initiation of T-cell dependent immune responses. IL-12 strongly influences the development of Th1 helper T-cell immune response, and induces cytotoxic T and NK cell function. An ELISA is used to measure the IL-12 release as follows. Dendritic cells (10^6/ml) are treated with increasing concentrations of an albumin fusion protein of the invention for 24 hours. LPS (100 ng/ml) is added to the cell culture as positive control. Supernatants from the cell cultures are then collected and analyzed for IL-12 content using commercial ELISA kit (e.g., R & D Systems (Minneapolis, MN)). The standard protocols provided with the kits are used.

Effect on the expression of MHC Class II, costimulatory and adhesion molecules. Three major families of cell surface antigens can be identified on monocytes: adhesion molecules, molecules involved in antigen presentation, and Fc receptor. Modulation of the expression of MHC class II antigens and other costimulatory molecules, such as B7 and ICAM-1, may result in changes in the antigen presenting capacity of monocytes and ability to

induce T cell activation. Increased expression of Fc receptors may correlate with improved monocyte cytotoxic activity, cytokine release and phagocytosis.

FACS analysis is used to examine the surface antigens as follows. Monocytes are treated 1-5 days with increasing concentrations of an albumin fusion protein of the invention or LPS (positive control), washed with PBS containing 1% BSA and 0.02 mM sodium azide, and then incubated with 1:20 dilution of appropriate FITC- or PE-labeled monoclonal antibodies for 30 minutes at 4 degrees C. After an additional wash, the labeled cells are analyzed by flow cytometry on a FACScan (Becton Dickinson).

Monocyte activation and/or increased survival. Assays for molecules that activate (or alternatively, inactivate) monocytes and/or increase monocyte survival (or alternatively, decrease monocyte survival) are known in the art and may routinely be applied to determine whether a molecule of the invention functions as an inhibitor or activator of monocytes. Albumin fusion proteins of the invention can be screened using the three assays described below. For each of these assays, Peripheral blood mononuclear cells (PBMC) are purified from single donor leukopacks (American Red Cross, Baltimore, MD) by centrifugation through a Histopaque gradient (Sigma). Monocytes are isolated from PBMC by counterflow centrifugal elutriation.

Monocyte Survival Assay. Human peripheral blood monocytes progressively lose viability when cultured in absence of serum or other stimuli. Their death results from internally regulated processes (apoptosis). Addition to the culture of activating factors, such as TNF-alpha dramatically improves cell survival and prevents DNA fragmentation. Propidium iodide (PI) staining is used to measure apoptosis as follows. Monocytes are cultured for 48 hours in polypropylene tubes in serum-free medium (positive control), in the presence of 100 ng/ml TNF-alpha (negative control), and in the presence of varying concentrations of the fusion protein to be tested. Cells are suspended at a concentration of 2 x 10^6/ml in PBS containing PI at a final concentration of 5 µg/ml, and then incubated at room temperature for 5 minutes before FACScan analysis. PI uptake has been demonstrated to correlate with DNA fragmentation in this experimental paradigm.

Effect on cytokine release. An important function of monocytes/macrophages is their regulatory activity on other cellular populations of the immune system through the release of cytokines after stimulation. An ELISA to measure cytokine release is performed as follows. Human monocytes are incubated at a density of $5x10^5$ cells/ml with increasing concentrations of an albumin fusion protein of the invention and under the same conditions, but in the

absence of the fusion protein. For IL-12 production, the cells are primed overnight with IFN (100 U/ml) in the presence of the fusion protein. LPS (10 ng/ml) is then added. Conditioned media are collected after 24h and kept frozen until use. Measurement of TNF-alpha, IL-10, MCP-1 and IL-8 is then performed using a commercially available ELISA kit (e.g., R & D Systems (Minneapolis, MN)) and applying the standard protocols provided with the kit.

Oxidative burst. Purified monocytes are plated in 96-w plate at 2-1x10^5 cell/well. Increasing concentrations of an albumin fusion protein of the invention are added to the wells in a total volume of 0.2 ml culture medium (RPMI 1640 + 10% FCS, glutamine and antibiotics). After 3 days incubation, the plates are centrifuged and the medium is removed from the wells. To the macrophage monolayers, 0.2 ml per well of phenol red solution (140 mM NaCl, 10 mM potassium phosphate buffer pH 7.0, 5.5 mM dextrose, 0.56 mM phenol red and 19 U/ml of HRPO) is added, together with the stimulant (200 nM PMA). The plates are incubated at 37°C for 2 hours and the reaction is stopped by adding 20 μl 1N NaOH per well. The absorbance is read at 610 nm. To calculate the amount of H_2O_2 produced by the macrophages, a standard curve of a H_2O_2 solution of known molarity is performed for each experiment.

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins or polynucleotides of the invention (e.g., gene therapy).

Example 23: Biological Effects of Fusion Proteins of the Invention

Astrocyte and Neuronal Assays:

Albumin fusion proteins of the invention can be tested for activity in promoting the survival, neurite outgrowth, or phenotypic differentiation of cortical neuronal cells and for inducing the proliferation of glial fibrillary acidic protein immunopositive cells, astrocytes. The selection of cortical cells for the bioassay is based on the prevalent expression of FGF-1 and FGF-2 in cortical structures and on the previously reported enhancement of cortical neuronal survival resulting from FGF-2 treatment. A thymidine incorporation assay, for example, can be used to elucidate an albumin fusion protein of the invention's activity on these cells.

Moreover, previous reports describing the biological effects of FGF-2 (basic FGF) on cortical or hippocampal neurons *in vitro* have demonstrated increases in both neuron survival and neurite outgrowth (Walicke et al., "Fibroblast growth factor promotes survival of dissociated hippocampal neurons and enhances neurite extension." *Proc. Natl. Acad. Sci.*

USA 83:3012-3016. (1986), assay herein incorporated by reference in its entirety). However, reports from experiments done on PC-12 cells suggest that these two responses are not necessarily synonymous and may depend on not only which FGF is being tested but also on which receptor(s) are expressed on the target cells. Using the primary cortical neuronal culture paradigm, the ability of an albumin fusion protein of the invention to induce neurite outgrowth can be compared to the response achieved with FGF-2 using, for example, a thymidine incorporation assay.

Fibroblast and endothelial cell assays·

Human lung fibroblasts are obtained from Clonetics (San Diego, CA) and maintained in growth media from Clonetics. Dermal microvascular endothelial cells are obtained from Cell Applications (San Diego, CA). For proliferation assays, the human lung fibroblasts and dermal microvascular endothelial cells can be cultured at 5,000 cells/well in a 96-well plate for one day in growth medium. The cells are then incubated for one day in 0.1% BSA basal medium. After replacing the medium with fresh 0.1% BSA medium, the cells are incubated with the test fusion protein of the invention proteins for 3 days. Alamar Blue (Alamar Biosciences, Sacramento, CA) is added to each well to a final concentration of 10%. The cells are incubated for 4 hr. Cell viability is measured by reading in a CytoFluor fluorescence reader. For the PGE_2 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or fusion protein of the invention with or without IL-1α for 24 hours. The supernatants are collected and assayed for PGE_2 by EIA kit (Cayman, Ann Arbor, MI). For the IL-6 assays, the human lung fibroblasts are cultured at 5,000 cells/well in a 96-well plate for one day. After a medium change to 0.1% BSA basal medium, the cells are incubated with FGF-2 or with or without an albumin fusion protein of the invention and/or IL-1α for 24 hours. The supernatants are collected and assayed for IL-6 by ELISA kit (Endogen, Cambridge, MA).

Human lung fibroblasts are cultured with FGF-2 or an albumin fusion protein of the invention for 3 days in basal medium before the addition of Alamar Blue to assess effects on growth of the fibroblasts. FGF-2 should show a stimulation at 10 - 2500 ng/ml which can be used to compare stimulation with the fusion protein of the invention.

Cell proliferation based on [3H]thymidine incorporation

The following [3H]Thymidine incorporation assay can be used to measure the effect

of a Therapeutic proteins, e.g., growth factor proteins, on the proliferation of cells such as fibroblast cells, epithelial cells or immature muscle cells.

Sub-confluent cultures are arrested in G1 phase by an 18 h incubation in serum-free medium. Therapeutic proteins are then added for 24 h and during the last 4 h, the cultures are labeled with [3H]thymidine, at a final concentration of 0.33 μM (25 Ci/mmol, Amersham, Arlington Heights, IL). The incorporated [3H]thymidine is precipitated with ice-cold 10% trichloroacetic acid for 24 h. Subsequently, the cells are rinsed sequentially with ice-cold 10% trichloroacetic acid and then with ice-cold water. Following lysis in 0.5 M NaOH, the lysates and PBS rinses (500 ml) are pooled, and the amount of radioactivity is measured.

Parkinson Models.

The loss of motor function in Parkinson's disease is attributed to a deficiency of striatal dopamine resulting from the degeneration of the nigrostriatal dopaminergic projection neurons. An animal model for Parkinson's that has been extensively characterized involves the systemic administration of 1-methyl-4 phenyl 1,2,3,6-tetrahydropyridine (MPTP). In the CNS, MPTP is taken-up by astrocytes and catabolized by monoamine oxidase B to 1-methyl-4-phenyl pyridine (MPP^+) and released. Subsequently, MPP^+ is actively accumulated in dopaminergic neurons by the high-affinity reuptake transporter for dopamine. MPP^+ is then concentrated in mitochondria by the electrochemical gradient and selectively inhibits nicotidamide adenine disphosphate: ubiquinone oxidoreductionase (complex I), thereby interfering with electron transport and eventually generating oxygen radicals.

It has been demonstrated in tissue culture paradigms that FGF-2 (basic FGF) has trophic activity towards nigral dopaminergic neurons (Ferrari et al., Dev. Biol. 1989). Recently, Dr. Unsicker's group has demonstrated that administering FGF-2 in gel foam implants in the striatum results in the near complete protection of nigral dopaminergic neurons from the toxicity associated with MPTP exposure (Otto and Unsicker, J. Neuroscience, 1990).

Based on the data with FGF-2, an albumin fusion protein of the invention can be evaluated to determine whether it has an action similar to that of FGF-2 in enhancing dopaminergic neuronal survival *in vitro* and it can also be tested *in vivo* for protection of dopaminergic neurons in the striatum from the damage associated with MPTP treatment. The potential effect of an albumin fusion protein of the invention is first examined in vitro in a dopaminergic neuronal cell culture paradigm. The cultures are prepared by dissecting the midbrain floor plate from gestation day 14 Wistar rat embryos. The tissue is dissociated with trypsin and seeded at a density of 200,000 cells/cm^2 on polyorthinine-laminin coated glass coverslips. The cells are maintained in Dulbecco's Modified Eagle's medium and F12 medium

containing hormonal supplements (N1). The cultures are fixed with paraformaldehyde after 8 days in vitro and are processed for tyrosine hydroxylase, a specific marker for dopaminergic neurons, immunohistochemical staining. Dissociated cell cultures are prepared from embryonic rats. The culture medium is changed every third day and the factors are also added at that time.

Since the dopaminergic neurons are isolated from animals at gestation day 14, a developmental time which is past the stage when the dopaminergic precursor cells are proliferating, an increase in the number of tyrosine hydroxylase immunopositive neurons would represent an increase in the number of dopaminergic neurons surviving *in vitro*. Therefore, if a Therapeutic protein acts to prolong the survival of dopaminergic neurons, it would suggest that the fusion protein may be involved in Parkinson's Disease.

The studies described in this example tested activity of albumin fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 24: The Effect of Albumin Fusion Proteins of the Invention on the Growth of Vascular Endothelial Cells

On day 1, human umbilical vein endothelial cells (HUVEC) are seeded at 2-5x10^4 cells/35 mm dish density in M199 medium containing 4% fetal bovine serum (FBS), 16 units/ml heparin, and 50 units/ml endothelial cell growth supplements (ECGS, Biotechnique, Inc.). On day 2, the medium is replaced with M199 containing 10% FBS, 8 units/ml heparin. An albumin fusion protein of the invention, and positive controls, such as VEGF and basic FGF (bFGF) are added, at varying concentrations. On days 4 and 6, the medium is replaced. On day 8, cell number is determined with a Coulter Counter.

An increase in the number of HUVEC cells indicates that the fusion protein may proliferate vascular endothelial cells, while a decrease in the number of HUVEC cells indicates that the fusion protein inhibits vascular endothelial cells.

The studies described in this example tested activity of an albumin fusion protein of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of a fusion protiem and polynucleotides of the invention.

Example 25: Rat Corneal Wound Healing Model

This animal model shows the effect of an albumin fusion protein of the invention on neovascularization. The experimental protocol includes:

Making a 1-1.5 mm long incision from the center of cornea into the stromal layer.

Inserting a spatula below the lip of the incision facing the outer corner of the eye.

Making a pocket (its base is 1-1.5 mm form the edge of the eye).

Positioning a pellet, containing 50ng- 5ug of an albumin fusion protein of the invention, within the pocket.

Treatment with an an albumin fusion protein of the invention can also be applied topically to the corneal wounds in a dosage range of 20mg - 500mg (daily treatment for five days).

The studies described in this example test the activity of an albumin fusion protein of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 26: Diabetic Mouse and Glucocorticoid-Impaired Wound Healing Models

Diabetic db+/db+ Mouse Model.

To demonstrate that an albumin fusion protein of the invention accelerates the healing process, the genetically diabetic mouse model of wound healing is used. The full thickness wound healing model in the db+/db+ mouse is a well characterized, clinically relevant and reproducible model of impaired wound healing. Healing of the diabetic wound is dependent on formation of granulation tissue and re-epithelialization rather than contraction (Gartner, M.H. *et al., J. Surg. Res. 52*:389 (1992); Greenhalgh, D.G. *et al., Am. J. Pathol. 136*:1235 (1990)).

The diabetic animals have many of the characteristic features observed in Type II diabetes mellitus. Homozygous (db+/db+) mice are obese in comparison to their normal heterozygous (db+/+m) littermates. Mutant diabetic (db+/db+) mice have a single autosomal recessive mutation on chromosome 4 (db+) (Coleman *et al. Proc. Natl. Acad. Sci. USA 77*:283-293 (1982)). Animals show polyphagia, polydipsia and polyuria. Mutant diabetic mice (db+/db+) have elevated blood glucose, increased or normal insulin levels, and suppressed cell-mediated immunity (Mandel *et al., J. Immunol. 120*:1375 (1978); Debray-Sachs, M. *et al., Clin. Exp. Immunol. 51(1)*:1-7 (1983); Leiter *et al., Am. J. of Pathol. 114*:46-55 (1985)). Peripheral neuropathy, myocardial complications, and microvascular lesions, basement membrane thickening and glomerular filtration abnormalities have been described in these animals (Norido, F. *et al., Exp. Neurol. 83(2)*:221-232 (1984); Robertson *et al., Diabetes 29(1)*:60-67 (1980); Giacomelli *et al., Lab Invest. 40(4)*:460-473 (1979); Coleman, D.L., *Diabetes 31 (Suppl)*:1-6 (1982)). These homozygous diabetic mice develop hyperglycemia that is resistant to insulin analogous to human type II diabetes (Mandel *et al., J. Immunol. 120*:1375-1377 (1978)).

The characteristics observed in these animals suggests that healing in this model may

be similar to the healing observed in human diabetes (Greenhalgh, *et al., Am. J. of Pathol. 136*:1235-1246 (1990)).

Genetically diabetic female C57BL/KsJ (db+/db+) mice and their non-diabetic (db+/+m) heterozygous littermates are used in this study (Jackson Laboratories). The animals are purchased at 6 weeks of age and are 8 weeks old at the beginning of the study. Animals are individually housed and received food and water ad libitum. All manipulations are performed using aseptic techniques. The experiments are conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

Wounding protocol is performed according to previously reported methods (Tsuboi, R. and Rifkin, D.B., *J. Exp. Med. 172*:245-251 (1990)). Briefly, on the day of wounding, animals are anesthetized with an intraperitoneal injection of Avertin (0.01 mg/mL), 2,2,2-tribromoethanol and 2-methyl-2-butanol dissolved in deionized water. The dorsal region of the animal is shaved and the skin washed with 70% ethanol solution and iodine. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is then created using a Keyes tissue punch. Immediately following wounding, the surrounding skin is gently stretched to eliminate wound expansion. The wounds are left open for the duration of the experiment. Application of the treatment is given topically for 5 consecutive days commencing on the day of wounding. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of surgery and at two day intervals thereafter. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

An albumin fusion protein of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology and immunohistochemistry. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Three groups of 10 animals each (5 diabetic and 5 non-diabetic controls) are evaluated: 1) Vehicle placebo control, 2) untreated group, and 3) treated group.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total square area of the wound. Contraction is then estimated by

establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm^2, the corresponding size of the dermal punch. Calculations are made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using a Reichert-Jung microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds are used to assess whether the healing process and the morphologic appearance of the repaired skin is altered by treatment with an albumin fusion protein of the invention. This assessment included verification of the presence of cell accumulation, inflammatory cells, capillaries, fibroblasts, re-epithelialization and epidermal maturity (Greenhalgh, D.G. *et al., Am. J. Pathol. 136:*1235 (1990)). A calibrated lens micrometer is used by a blinded observer.

Tissue sections are also stained immunohistochemically with a polyclonal rabbit anti-human keratin antibody using ABC Elite detection system. Human skin is used as a positive tissue control while non-immune IgG is used as a negative control. Keratinocyte growth is determined by evaluating the extent of reepithelialization of the wound using a calibrated lens micrometer.

Proliferating cell nuclear antigen/cyclin (PCNA) in skin specimens is demonstrated by using anti-PCNA antibody (1:50) with an ABC Elite detection system. Human colon cancer served as a positive tissue control and human brain tissue is used as a negative tissue control. Each specimen included a section with omission of the primary antibody and substitution with non-immune mouse IgG. Ranking of these sections is based on the extent of proliferation on a scale of 0-8, the lower side of the scale reflecting slight proliferation to the higher side reflecting intense proliferation.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

Steroid Impaired Rat Model

The inhibition of wound healing by steroids has been well documented in various *in vitro* and *in vivo* systems (Wahl, Glucocorticoids and Wound healing. In: Anti-Inflammatory Steroid Action: Basic and Clinical Aspects. 280-302 (1989); Wahl*et al., J. Immunol. 115*: 476-481 (1975); Werb *et al., J. Exp. Med. 147*:1684-1694 (1978)). Glucocorticoids retard wound healing by inhibiting angiogenesis, decreasing vascular permeability (Ebert *et al., An.*

Intern. Med. 37:701-705 (1952)), fibroblast proliferation, and collagen synthesis (Beck *et al.*, *Growth Factors. 5:* 295-304 (1991); Haynes *et al.*, *J. Clin. Invest. 61:* 703-797 (1978)) and producing a transient reduction of circulating monocytes (Haynes et al., *J. Clin. Invest. 61:* 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In:* Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989)). The systemic administration of steroids to impaired wound healing is a well establish phenomenon in rats (Beck *et al.*, *Growth Factors. 5:* 295-304 (1991); Haynes *et al.*, *J. Clin. Invest. 61:* 703-797 (1978); Wahl, "Glucocorticoids and wound healing", *In:* Antiinflammatory Steroid Action: Basic and Clinical Aspects, Academic Press, New York, pp. 280-302 (1989); Pierce *et al.*, *Proc. Natl. Acad. Sci. USA 86*: 2229-2233 (1989)).

To demonstrate that an albumin fusion protein of the invention can accelerate the healing process, the effects of multiple topical applications of the fusion protein on full thickness excisional skin wounds in rats in which healing has been impaired by the systemic administration of methylprednisolone is assessed.

Young adult male Sprague Dawley rats weighing 250-300 g (Charles River Laboratories) are used in this example. The animals are purchased at 8 weeks of age and are 9 weeks old at the beginning of the study. The healing response of rats is impaired by the systemic administration of methylprednisolone (17mg/kg/rat intramuscularly) at the time of wounding. Animals are individually housed and received food and water *ad libitum*. All manipulations are performed using aseptic techniques. This study is conducted according to the rules and guidelines of Human Genome Sciences, Inc. Institutional Animal Care and Use Committee and the Guidelines for the Care and Use of Laboratory Animals.

The wounding protocol is followed according to that described above. On the day of wounding, animals are anesthetized with an intramuscular injection of ketamine-(50 mg/kg) and xylazine (5 mg/kg). The dorsal region of the animal is shaved and the skin washed with 70% ethanol and iodine solutions. The surgical area is dried with sterile gauze prior to wounding. An 8 mm full-thickness wound is created using a Keyes tissue punch. The wounds are left open for the duration of the experiment. Applications of the testing materials are given topically once a day for 7 consecutive days commencing on the day of wounding and subsequent to methylprednisolone administration. Prior to treatment, wounds are gently cleansed with sterile saline and gauze sponges.

Wounds are visually examined and photographed at a fixed distance at the day of wounding and at the end of treatment. Wound closure is determined by daily measurement on days 1-5 and on day 8. Wounds are measured horizontally and vertically using a calibrated Jameson caliper. Wounds are considered healed if granulation tissue is no longer visible and the wound is covered by a continuous epithelium.

The fusion protein of the invention is administered using at a range different doses, from 4mg to 500mg per wound per day for 8 days in vehicle. Vehicle control groups received 50mL of vehicle solution.

Animals are euthanized on day 8 with an intraperitoneal injection of sodium pentobarbital (300mg/kg). The wounds and surrounding skin are then harvested for histology. Tissue specimens are placed in 10% neutral buffered formalin in tissue cassettes between biopsy sponges for further processing.

Three groups of 10 animals each (5 with methylprednisolone and 5 without glucocorticoid) are evaluated: 1) Untreated group 2) Vehicle placebo control 3) treated groups.

Wound closure is analyzed by measuring the area in the vertical and horizontal axis and obtaining the total area of the wound. Closure is then estimated by establishing the differences between the initial wound area (day 0) and that of post treatment (day 8). The wound area on day 1 is 64mm^2, the corresponding size of the dermal punch. Calculations are made using the following formula:

[Open area on day 8] - [Open area on day 1] / [Open area on day 1]

Specimens are fixed in 10% buffered formalin and paraffin embedded blocks are sectioned perpendicular to the wound surface (5mm) and cut using an Olympus microtome. Routine hematoxylin-eosin (H&E) staining is performed on cross-sections of bisected wounds. Histologic examination of the wounds allows assessment of whether the healing process and the morphologic appearance of the repaired skin is improved by treatment with an albumin fusion protein of the invention. A calibrated lens micrometer is used by a blinded observer to determine the distance of the wound gap.

Experimental data are analyzed using an unpaired t test. A p value of < 0.05 is considered significant.

The studies described in this example tested activity of an albumin fusion protein of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 27: Lymphedema Animal Model

The purpose of this experimental approach is to create an appropriate and consistent lymphedema model for testing the therapeutic effects of an albumin fusion protein of the invention in lymphangiogenesis and re-establishment of the lymphatic circulatory system in the rat hind limb. Effectiveness is measured by swelling volume of the affected limb, quantification of the amount of lymphatic vasculature, total blood plasma protein, and

histopathology. Acute lymphedema is observed for 7-10 days. Perhaps more importantly, the chronic progress of the edema is followed for up to 3-4 weeks.

Prior to beginning surgery, blood sample is drawn for protein concentration analysis. Male rats weighing approximately ~350g are dosed with Pentobarbital. Subsequently, the right legs are shaved from knee to hip. The shaved area is swabbed with gauze soaked in 70% EtOH. Blood is drawn for serum total protein testing. Circumference and volumetric measurements are made prior to injecting dye into paws after marking 2 measurement levels (0.5 cm above heel, at mid-pt of dorsal paw). The intradermal dorsum of both right and left paws are injected with 0.05 ml of 1% Evan's Blue. Circumference and volumetric measurements are then made following injection of dye into paws.

Using the knee joint as a landmark, a mid-leg inguinal incision is made circumferentially allowing the femoral vessels to be located. Forceps and hemostats are used to dissect and separate the skin flaps. After locating the femoral vessels, the lymphatic vessel that runs along side and underneath the vessel(s) is located. The main lymphatic vessels in this area are then electrically coagulated or suture ligated.

Using a microscope, muscles in back of the leg (near the semitendinosis and adductors) are bluntly dissected. The popliteal lymph node is then located. The 2 proximal and 2 distal lymphatic vessels and distal blood supply of the popliteal node are then ligated by suturing. The popliteal lymph node, and any accompanying adipose tissue, is then removed by cutting connective tissues.

Care is taken to control any mild bleeding resulting from this procedure. After lymphatics are occluded, the skin flaps are sealed by using liquid skin (Vetbond) (AJ Buck). The separated skin edges are sealed to the underlying muscle tissue while leaving a gap of ~0.5 cm around the leg. Skin also may be anchored by suturing to underlying muscle when necessary.

To avoid infection, animals are housed individually with mesh (no bedding). Recovering animals are checked daily through the optimal edematous peak, which typically occurred by day 5-7. The plateau edematous peak are then observed. To evaluate the intensity of the lymphedema, the circumference and volumes of 2 designated places on each paw before operation and daily for 7 days are measured. The effect of plasma proteins on lymphedema is determined and whether protein analysis is a useful testing perimeter is also investigated. The weights of both control and edematous limbs are evaluated at 2 places. Analysis is performed in a blind manner.

Circumference Measurements: Under brief gas anesthetic to prevent limb movement, a cloth tape is used to measure limb circumference. Measurements are done at the ankle bone and dorsal paw by 2 different people and those 2 readings are averaged. Readings are taken

from both control and edematous limbs.

Volumetric Measurements: On the day of surgery, animals are anesthetized with Pentobarbital and are tested prior to surgery. For daily volumetrics animals are under brief halothane anesthetic (rapid immobilization and quick recovery), and both legs are shaved and equally marked using waterproof marker on legs. Legs are first dipped in water, then dipped into instrument to each marked level then measured by Buxco edema software(Chen/Victor). Data is recorded by one person, while the other is dipping the limb to marked area.

Blood-plasma protein measurements: Blood is drawn, spun, and serum separated prior to surgery and then at conclusion for total protein and $Ca2^{+}$ comparison.

Limb Weight Comparison: After drawing blood, the animal is prepared for tissue collection. The limbs are amputated using a quillitine, then both experimental and control legs are cut at the ligature and weighed. A second weighing is done as the tibio-cacaneal joint is disarticulated and the foot is weighed.

Histological Preparations: The transverse muscle located behind the knee (popliteal) area is dissected and arranged in a metal mold, filled with freezeGel, dipped into cold methylbutane, placed into labeled sample bags at - 80EC until sectioning. Upon sectioning, the muscle is observed under fluorescent microscopy for lymphatics..

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion protein and polynucleotides of the invention (e.g., gene therapy).

Example 28: Suppression of TNF alpha-Induced Adhesion Molecule Expression by an Albumin Fusion Protein of the Invention

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

Tumor necrosis factor alpha (TNF-a), a potent proinflammatory cytokine, is a stimulator of all three CAMs on endothelial cells and may be involved in a wide variety of inflammatory responses, often resulting in a pathological outcome.

The potential of an albumin fusion protein of the invention to mediate a suppression of TNF-a induced CAM expression can be examined. A modified ELISA assay which uses ECs as a solid phase absorbent is employed to measure the amount of CAM expression on TNF-a treated ECs when co-stimulated with a member of the FGF family of proteins.

To perform the experiment, human umbilical vein endothelial cell (HUVEC) cultures are obtained from pooled cord harvests and maintained in growth medium (EGM-2; Clonetics, San Diego, CA) supplemented with 10% FCS and 1% penicillin/streptomycin in a 37 degree C humidified incubator containing 5% CO_2. HUVECs are seeded in 96-well plates at concentrations of 1 x 10^4 cells/well in EGM medium at 37 degree C for 18-24 hrs or until confluent. The monolayers are subsequently washed 3 times with a serum-free solution of RPMI-1640 supplemented with 100 U/ml penicillin and 100 mg/ml streptomycin, and treated with a given cytokine and/or growth factor(s) for 24 h at 37 degree C. Following incubation, the cells are then evaluated for CAM expression.

Human Umbilical Vein Endothelial cells (HUVECs) are grown in a standard 96 well plate to confluence. Growth medium is removed from the cells and replaced with 90 ul of 199 Medium (10% FBS). Samples for testing and positive or negative controls are added to the plate in triplicate (in 10 ul volumes). Plates are incubated at 37 degree C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μl of 0.1% paraformaldehyde-PBS(with Ca++ and Mg++) is added to each well. Plates are held at 4°C for 30 min.

Fixative is then removed from the wells and wells are washed 1X with PBS(+Ca,Mg)+0.5% BSA and drained. Do not allow the wells to dry. Add 10 μl of diluted primary antibody to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μg/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA.

Then add 20 μl of diluted ExtrAvidin-Alkaline Phosphotase (1:5,000 dilution) to each well and incubated at 37°C for 30 min. Wells are washed X3 with PBS(+Ca,Mg)+0.5% BSA. 1 tablet of p-Nitrophenol Phosphate pNPP is dissolved in 5 ml of glycine buffer (pH 10.4). 100 μl of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphotase in glycine buffer: 1:5,000 (10^0) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μl of pNNP reagent must then be added to each of the standard wells. The plate must be incubated at 37°C for 4h. A volume of 50 μl of 3M NaOH is added to all wells. The results are quantified on a plate reader at 405 nm. The background subtraction option is used on

blank wells filled with glycine buffer only. The template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

The studies described in this example tested activity of fusion proteins of the invention. However, one skilled in the art could easily modify the exemplified studies to test the activity of fusion proteins and polynucleotides of the invention (e.g., gene therapy).

Example 29: Construction of GAS Reporter Construct

One signal transduction pathway involved in the differentiation and proliferation of cells is called the Jaks-STATs pathway. Activated proteins in the Jaks-STATs pathway bind to gamma activation site "GAS" elements or interferon-sensitive responsive element ("ISRE"), located in the promoter of many genes. The binding of a protein to these elements alter the expression of the associated gene.

GAS and ISRE elements are recognized by a class of transcription factors called Signal Transducers and Activators of Transcription, or "STATs." There are six members of the STATs family. Stat1 and Stat3 are present in many cell types, as is Stat2 (as response to IFN-alpha is widespread). Stat4 is more restricted and is not in many cell types though it has been found in T helper class I, cells after treatment with IL-12. Stat5 was originally called mammary growth factor, but has been found at higher concentrations in other cells including myeloid cells. It can be activated in tissue culture cells by many cytokines.

The STATs are activated to translocate from the cytoplasm to the nucleus upon tyrosine phosphorylation by a set of kinases known as the Janus Kinase ("Jaks") family. Jaks represent a distinct family of soluble tyrosine kinases and include Tyk2, Jak1, Jak2, and Jak3. These kinases display significant sequence similarity and are generally catalytically inactive in resting cells.

The Jaks are activated by a wide range of receptors summarized in the Table below. (Adapted from review by Schidler and Darnell, Ann. Rev. Biochem. 64:621-51 (1995)). A cytokine receptor family, capable of activating Jaks, is divided into two groups: (a) Class 1 includes receptors for IL-2, IL-3, IL-4, IL-6, IL-7, IL-9, IL-11, IL-12, IL-15, Epo, PRL, GH, G-CSF, GM-CSF, LIF, CNTF, and thrombopoietin; and (b) Class 2 includes IFN-a, IFN-g, and IL-10. The Class 1 receptors share a conserved cysteine motif (a set of four conserved cysteines and one tryptophan) and a WSXWS motif (a membrane proximal region encoding Trp-Ser-Xaa-Trp-Ser (SEQ ID NO: 37)).

Thus, on binding of a ligand to a receptor, Jaks are activated, which in turn activate STATs, which then translocate and bind to GAS elements. This entire process is encompassed in the Jaks-STATs signal transduction pathway. Therefore, activation of the

Jaks-STATs pathway, reflected by the binding of the GAS or the ISRE element, can be used to indicate proteins involved in the proliferation and differentiation of cells. For example, growth factors and cytokines are known to activate the Jaks-STATs pathway (See Table below). Thus, by using GAS elements linked to reporter molecules, activators of the Jaks-STATs pathway can be identified.

Ligand	JAKs tyk2	Jak1	Jak2	Jak3	STATS	GAS(elements) or ISRE
IFN family						
IFN-a/B	+	+	-	-	1,2,3	ISRE
IFN-g		+	+	-	1	GAS (IRF1>Lys6>IFP)
Il-10	+	?	?	-	1,3	
gp130 family						
IL-6 (Pleiotropic)	+	+	+	?	1,3	GAS (IRF1>Lys6>IFP)
Il-11(Pleiotropic)	?	+	?	?	1,3	
OnM(Pleiotropic)	?	+	+	?	1,3	
LIF(Pleiotropic)	?	+	+	?	1,3	
CNTF(Pleiotropic)	-/+	+	+	?	1,3	
G-CSF(Pleiotropic)	?	+	?	?	1,3	
IL-12(Pleiotropic)	+	-	+	+	1,3	
g-C family						
IL-2 (lymphocytes)	-	+	-	+	1,3,5	GAS
IL-4 (lymph/myeloid) >>Ly6)(IgH)	-	+	-	+	6	GAS(IRF1=IFP
IL-7 (lymphocytes)	-	+	-	+	5	GAS
IL-9 (lymphocytes)	-	+	-	+	5	GAS
IL-13 (lymphocyte)	-	+	?	?	6	GAS
IL-15	?	+	?	+	5	GAS
gp140 family						
IL-3 (myeloid)	-	-	+	-	5	GAS (IRF1>IFP>>Ly6)
IL-5 (myeloid)	-	-	+	-	5	GAS
GM-CSF (myeloid)	-	-	+	-	5	GAS
Growth hormone family						
GH	?	-	+	-	5	
PRL	?	+/-	+	-	1,3,5	
EPO	?	-	+	-	5	GAS(B-CAS>IRF1=IFP>>Ly6)
Receptor Tyrosine Kinases						
EGF	?	+	+	-	1,3	GAS (IRF1)
PDGF	?	+	+	-	1,3	
CSF-1	?	+	+	-	1,3	GAS (not IRF1)

To construct a synthetic GAS containing promoter element, which is used in the Biological Assays described in Examples 32-33, a PCR based strategy is employed to generate a GAS-SV40 promoter sequence. The 5' primer contains four tandem copies of the GAS binding site found in the IRF1 promoter and previously demonstrated to bind STATs upon induction with a range of cytokines (Rothman et al., Immunity 1:457-468 (1994).), although other GAS or ISRE elements can be used instead. The 5' primer also contains 18bp of sequence complementary to the SV40 early promoter sequence and is flanked with an XhoI site. The sequence of the 5' primer is:

5':GCGCCTCGAGATTTCCCCGAAATCTAGATTTCCCCGAAATGATTTCCCC GAAATGATTTCCCCGAAATATCTGCCATCTCAATTAG:3' (SEQ ID NO: 38)

The downstream primer is complementary to the SV40 promoter and is flanked with a Hind III site: 5':GCGGCAAGCTTTTTGCAAAGCCTAGGC:3' (SEQ ID NO: 39)

PCR amplification is performed using the SV40 promoter template present in the B-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI/Hind III and subcloned into BLSK2-. (Stratagene.) Sequencing with forward and reverse primers confirms that the insert contains the following sequence:

5':CTCGAGATTTCCCCGAAATCTAGATTTCCCCGAAATGATTTCCCCGAAAT GATTTCCCCGAAATATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTA ACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATG GCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTA TTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTT:3' (SEQ ID NO: 40)

With this GAS promoter element linked to the SV40 promoter, a GAS:SEAP2 reporter construct is next engineered. Here, the reporter molecule is a secreted alkaline phosphatase, or "SEAP." Clearly, however, any reporter molecule can be instead of SEAP, in this or in any of the other Examples. Well known reporter molecules that can be used instead of SEAP include chloramphenicol acetyltransferase (CAT), luciferase, alkaline phosphatase, B-galactosidase, green fluorescent protein (GFP), or any protein detectable by an antibody.

The above sequence confirmed synthetic GAS-SV40 promoter element is subcloned into the pSEAP-Promoter vector obtained from Clontech using HindIII and XhoI, effectively replacing the SV40 promoter with the amplified GAS:SV40 promoter element, to create the GAS-SEAP vector. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

Thus, in order to generate mammalian stable cell lines expressing the GAS-SEAP reporter, the GAS-SEAP cassette is removed from the GAS-SEAP vector using SalI and

NotI, and inserted into a backbone vector containing the neomycin resistance gene, such as pGFP-1 (Clontech), using these restriction sites in the multiple cloning site, to create the GAS-SEAP/Neo vector. Once this vector is transfected into mammalian cells, this vector can then be used as a reporter molecule for GAS binding as described in Examples 32-33.

Other constructs can be made using the above description and replacing GAS with a different promoter sequence. For example, construction of reporter molecules containing EGR and NF-KB promoter sequences are described in Examples 34 and 35. However, many other promoters can be substituted using the protocols described in these Examples. For instance, SRE, IL-2, NFAT, or Osteocalcin promoters can be substituted, alone or in combination (e.g., GAS/NF-KB/EGR, GAS/NF-KB, Il-2/NFAT, or NF-KB/GAS). Similarly, other cell lines can be used to test reporter construct activity, such as HELA (epithelial), HUVEC (endothelial), Reh (B-cell), Saos-2 (osteoblast), HUVAC (aortic), or Cardiomyocyte.

Example 30: Assay for SEAP Activity

As a reporter molecule for the assays described in examples disclosed herein, SEAP activity is assayed using the Tropix Phospho-light Kit (Cat. BP-400) according to the following general procedure. The Tropix Phospho-light Kit supplies the Dilution, Assay, and Reaction Buffers used below.

Prime a dispenser with the 2.5x Dilution Buffer and dispense 15 ul of 2.5x dilution buffer into Optiplates containing 35 ul of a solution containing an albumin fusion protein of the invention. Seal the plates with a plastic sealer and incubate at 65 degree C for 30 min. Separate the Optiplates to avoid uneven heating.

Cool the samples to room temperature for 15 minutes. Empty the dispenser and prime with the Assay Buffer. Add 50 ml Assay Buffer and incubate at room temperature 5 min. Empty the dispenser and prime with the Reaction Buffer (see the Table below). Add 50 ul Reaction Buffer and incubate at room temperature for 20 minutes. Since the intensity of the chemiluminescent signal is time dependent, and it takes about 10 minutes to read 5 plates on a luminometer, thus one should treat 5 plates at each time and start the second set 10 minutes later.

Read the relative light unit in the luminometer. Set H12 as blank, and print the results. An increase in chemiluminescence indicates reporter activity.

Reaction Buffer Formulation:

# of plates	Rxn buffer diluent (ml)	CSPD (ml)
10	60	3
11	65	3.25

12	70	3.5
13	75	3.75
14	80	4
15	85	4.25
16	90	4.5
17	95	4.75
18	100	5
19	105	5.25
20	110	5.5
21	115	5.75
22	120	6
23	125	6.25
24	130	6.5
25	135	6.75
26	140	7
27	145	7.25
28	150	7.5
29	155	7.75
30	160	8
31	165	8.25
32	170	8.5
33	175	8.75
34	180	9
35	185	9.25
36	190	9.5
37	195	9.75
38	200	10
39	205	10.25
40	210	10.5
41	215	10.75
42	220	11
43	225	11.25
44	230	11.5
45	235	11.75
46	240	12
47	245	12.25
48	250	12.5
49	255	12.75
50	260	13

Example 31: Assay Identifying Neuronal Activity.

When cells undergo differentiation and proliferation, a group of genes are activated through many different signal transduction pathways. One of these genes, EGR1 (early growth response gene 1), is induced in various tissues and cell types upon activation. The promoter of EGR1 is responsible for such induction. Using the EGR1 promoter linked to reporter molecules, the ability of fusion proteins of the invention to activate cells can be assessed.

Particularly, the following protocol is used to assess neuronal activity in PC12 cell

lines. PC12 cells (rat phenochromocytoma cells) are known to proliferate and/or differentiate by activation with a number of mitogens, such as TPA (tetradecanoyl phorbol acetate), NGF (nerve growth factor), and EGF (epidermal growth factor). The EGR1 gene expression is activated during this treatment. Thus, by stably transfecting PC12 cells with a construct containing an EGR promoter linked to SEAP reporter, activation of PC12 cells by an albumin fusion protein of the present invention can be assessed.

The EGR/SEAP reporter construct can be assembled by the following protocol. The EGR-1 promoter sequence (-633 to +1)(Sakamoto K et al., Oncogene 6:867-871 (1991)) can be PCR amplified from human genomic DNA using the following primers:

5' GCGCTCGAGGGATGACAGCGATAGAACCCCGG-3' (SEQ ID NO: 41)

5' GCGAAGCTTCGCGACTCCCCGGATCCGCCTC-3' (SEQ ID NO: 42)

Using the GAS:SEAP/Neo vector produced in Example 29, EGR1 amplified product can then be inserted into this vector. Linearize the GAS:SEAP/Neo vector using restriction enzymes XhoI/HindIII, removing the GAS/SV40 stuffer. Restrict the EGR1 amplified product with these same enzymes. Ligate the vector and the EGR1 promoter.

To prepare 96 well-plates for cell culture, two mls of a coating solution (1:30 dilution of collagen type I (Upstate Biotech Inc. Cat#08-115) in 30% ethanol (filter sterilized)) is added per one 10 cm plate or 50 ml per well of the 96-well plate, and allowed to air dry for 2 hr.

PC12 cells are routinely grown in RPMI-1640 medium (Bio Whittaker) containing 10% horse serum (JRH BIOSCIENCES, Cat. # 12449-78P), 5% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 ug/ml streptomycin on a precoated 10 cm tissue culture dish. One to four split is done every three to four days. Cells are removed from the plates by scraping and resuspended with pipetting up and down for more than 15 times.

Transfect the EGR/SEAP/Neo construct into PC12 using techniques known in the art. EGR-SEAP/PC12 stable cells are obtained by growing the cells in 300 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 300 ug/ml G418 for couple of passages.

To assay for neuronal activity, a 10 cm plate with cells around 70 to 80% confluent is screened by removing the old medium. Wash the cells once with PBS (Phosphate buffered saline). Then starve the cells in low serum medium (RPMI-1640 containing 1% horse serum and 0.5% FBS with antibiotics) overnight.

The next morning, remove the medium and wash the cells with PBS. Scrape off the cells from the plate, suspend the cells well in 2 ml low serum medium. Count the cell number and add more low serum medium to reach final cell density as $5x10^5$ cells/ml.

Add 200 ul of the cell suspension to each well of 96-well plate (equivalent to $1x10^5$ cells/well). Add a series of different concentrations of an albumin fusion protein of the inventon, 37 degree C for 48 to 72 hr. As a positive control, a growth factor known to activate PC12 cells through EGR can be used, such as 50 ng/ul of Neuronal Growth Factor (NGF). Over fifty-fold induction of SEAP is typically seen in the positive control wells. SEAP assay may be routinely performed using techniques known in the art and/or as described in Example 30.

Example 32: Assay for T-cell Activity.

The following protocol is used to assess T-cell activity by identifying factors, and determining whether an albumin fusion protein of the invention proliferates and/or differentiates T-cells. T-cell activity is assessed using the GAS/SEAP/Neo construct produced in Example 29. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The T-cell used in this assay is Jurkat T-cells (ATCC Accession No. TIB-152), although Molt-3 cells (ATCC Accession No. CRL-1552) and Molt-4 cells (ATCC Accession No. CRL-1582) cells can also be used.

Jurkat T-cells are lymphoblastic CD4+ Th1 helper cells. In order to generate stable cell lines, approximately 2 million Jurkat cells are transfected with the GAS-SEAP/neo vector using DMRIE-C (Life Technologies)(transfection procedure described below). The transfected cells are seeded to a density of approximately 20,000 cells per well and transfectants resistant to 1 mg/ml genticin selected. Resistant colonies are expanded and then tested for their response to increasing concentrations of interferon gamma. The dose response of a selected clone is demonstrated.

Specifically, the following protocol will yield sufficient cells for 75 wells containing 200 ul of cells. Thus, it is either scaled up, or performed in multiple to generate sufficient cells for multiple 96 well plates. Jurkat cells are maintained in RPMI + 10% serum with 1%Pen-Strep. Combine 2.5 mls of OPTI-MEM (Life Technologies) with 10 ug of plasmid DNA in a T25 flask. Add 2.5 ml OPTI-MEM containing 50 ul of DMRIE-C and incubate at room temperature for 15-45 mins.

During the incubation period, count cell concentration, spin down the required number of cells (10^7 per transfection), and resuspend in OPTI-MEM to a final concentration of 10^7 cells/ml. Then add 1ml of 1 x 10^7 cells in OPTI-MEM to T25 flask and incubate at 37 degree C for 6 hrs. After the incubation, add 10 ml of RPMI + 15% serum.

The Jurkat:GAS-SEAP stable reporter lines are maintained in RPMI + 10% serum, 1 mg/ml Genticin, and 1% Pen-Strep. These cells are treated with varying concentrations of one or more fusion proteins of the present invention.

On the day of treatment with the fusion protein, the cells should be washed and resuspended in fresh RPMI + 10% serum to a density of 500,000 cells per ml. The exact number of cells required will depend on the number of fusion proteins and the number of different concentrations of fusion proteins being screened. For one 96 well plate, approximately 10 million cells (for 10 plates, 100 million cells) are required.

The well dishes containing Jurkat cells treated with the fusion protein are placed in an incubator for 48 hrs (note: this time is variable between 48-72 hrs). 35 ul samples from each well are then transferred to an opaque 96 well plate using a 12 channel pipette. The opaque plates should be covered (using sellophene covers) and stored at -20 degree C until SEAP assays are performed according to Example 30. The plates containing the remaining treated cells are placed at 4 degree C and serve as a source of material for repeating the assay on a specific well if desired.

As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate Jurkat T cells. Over 30 fold induction is typically observed in the positive control wells.

The above protocol may be used in the generation of both transient, as well as, stable transfected cells, which would be apparent to those of skill in the art.

Example 33: Assay for T-cell Activity

NF-KB (Nuclear Factor KB) is a transcription factor activated by a wide variety of agents including the inflammatory cytokines IL-1 and TNF, CD30 and CD40, lymphotoxin-alpha and lymphotoxin-beta, by exposure to LPS or thrombin, and by expression of certain viral gene products. As a transcription factor, NF-KB regulates the expression of genes involved in immune cell activation, control of apoptosis (NF- KB appears to shield cells from apoptosis), B and T-cell development, anti-viral and antimicrobial responses, and multiple stress responses.

In non-stimulated conditions, NF- KB is retained in the cytoplasm with I-KB (Inhibitor KB). However, upon stimulation, I- KB is phosphorylated and degraded, causing NF- KB to shuttle to the nucleus, thereby activating transcription of target genes. Target genes activated by NF- KB include IL-2, IL-6, GM-CSF, ICAM-1 and class 1 MHC.

Due to its central role and ability to respond to a range of stimuli, reporter constructs utilizing the NF-KB promoter element are used to screen the fusion protein. Activators or inhibitors of NF-KB would be useful in treating, preventing, and/or diagnosing diseases. For example, inhibitors of NF-KB could be used to treat those diseases related to the acute or chronic activation of NF-KB, such as rheumatoid arthritis.

To construct a vector containing the NF-KB promoter element, a PCR based strategy

is employed. The upstream primer contains four tandem copies of the NF-KB binding site (GGGGACTTTCCC) (SEQ ID NO: 43), 18 bp of sequence complementary to the 5' end of the SV40 early promoter sequence, and is flanked with an XhoI site:

5':GCGGCCTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGA CTTTCCATCCTGCCATCTCAATTAG:3' (SEQ ID NO: 44)

The downstream primer is complementary to the 3' end of the SV40 promoter and is flanked with a Hind III site:

5':GCGGCAAGCTTTTTGCAAAGCCTAGGC:3' (SEQ ID NO: 39)

PCR amplification is performed using the SV40 promoter template present in the pB-gal:promoter plasmid obtained from Clontech. The resulting PCR fragment is digested with XhoI and Hind III and subcloned into BLSK2-. (Stratagene) Sequencing with the T7 and T3 primers confirms the insert contains the following sequence:

5':CTCGAGGGGACTTTCCCGGGGACTTTCCGGGGACTTTCCGGGACTTTCC ATCTGCCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCC GCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTT TTATTTATGCAGAGGCCGAGGCCGCCTCGGCCTCTGAGCTATTCCAGAAGTAGTG AGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAAAGCTT:3' (SEQ ID NO: 45)

Next, replace the SV40 minimal promoter element present in the pSEAP2-promoter plasmid (Clontech) with this NF-KB/SV40 fragment using XhoI and HindIII. However, this vector does not contain a neomycin resistance gene, and therefore, is not preferred for mammalian expression systems.

In order to generate stable mammalian cell lines, the NF-KB/SV40/SEAP cassette is removed from the above NF-KB/SEAP vector using restriction enzymes SalI and NotI, and inserted into a vector containing neomycin resistance. Particularly, the NF-KB/SV40/SEAP cassette was inserted into pGFP-1 (Clontech), replacing the GFP gene, after restricting pGFP-1 with SalI and NotI.

Once NF-KB/SV40/SEAP/Neo vector is created, stable Jurkat T-cells are created and maintained according to the protocol described in Example 32. Similarly, the method for assaying fusion proteins with these stable Jurkat T-cells is also described in Example 32. As a positive control, exogenous TNF alpha (0.1,1, 10 ng) is added to wells H9, H10, and H11, with a 5-10 fold activation typically observed.

Example 33: Assay Identifying Myeloid Activity

The following protocol is used to assess myeloid activity of an albumin fusion protein of the present invention by determining whether the fusion protein proliferates and/or differentiates myeloid cells. Myeloid cell activity is assessed using the GAS/SEAP/Neo

construct produced in Example 29. Thus, factors that increase SEAP activity indicate the ability to activate the Jaks-STATS signal transduction pathway. The myeloid cell used in this assay is U937, a pre-monocyte cell line, although TF-1, HL60, or KG1 can be used.

To transiently transfect U937 cells with the GAS/SEAP/Neo construct produced in Example 29, a DEAE-Dextran method (Kharbanda et. al., 1994, Cell Growth & Differentiation, 5:259-265) is used. First, harvest $2x10^7$ U937 cells and wash with PBS. The U937 cells are usually grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum (FBS) supplemented with 100 units/ml penicillin and 100 mg/ml streptomycin.

Next, suspend the cells in 1 ml of 20 mM Tris-HCl (pH 7.4) buffer containing 0.5 mg/ml DEAE-Dextran, 8 ug GAS-SEAP2 plasmid DNA, 140 mM NaCl, 5 mM KCl, 375 uM $Na_2HPO_4.7H_2O$, 1 mM $MgCl_2$, and 675 uM $CaCl_2$. Incubate at 37 degrees C for 45 min.

Wash the cells with RPMI 1640 medium containing 10% FBS and then resuspend in 10 ml complete medium and incubate at 37 degree C for 36 hr.

The GAS-SEAP/U937 stable cells are obtained by growing the cells in 400 ug/ml G418. The G418-free medium is used for routine growth but every one to two months, the cells should be re-grown in 400 ug/ml G418 for couple of passages.

These cells are tested by harvesting $1x10^8$ cells (this is enough for ten 96-well plates assay) and wash with PBS. Suspend the cells in 200 ml above described growth medium, with a final density of $5x10^5$ cells/ml. Plate 200 ul cells per well in the 96-well plate (or $1x10^5$ cells/well).

Add different concentrations of the fusion protein. Incubate at 37 degee C for 48 to 72 hr. As a positive control, 100 Unit/ml interferon gamma can be used which is known to activate U937 cells. Over 30 fold induction is typically observed in the positive control wells. SEAP assay the supernatant according to methods known in the art and/or the protocol described in Example 30.

Example 34: Assay Identifying Changes in Small Molecule Concentration and Membrane Permeability

Binding of a ligand to a receptor is known to alter intracellular levels of small molecules, such as calcium, potassium, sodium, and pH, as well as alter membrane potential. These alterations can be measured in an assay to identify fusion proteins which bind to receptors of a particular cell. Although the following protocol describes an assay for calcium, this protocol can easily be modified to detect changes in potassium, sodium, pH, membrane potential, or any other small molecule which is detectable by a fluorescent probe.

The following assay uses Fluorometric Imaging Plate Reader ("FLIPR") to measure

changes in fluorescent molecules (Molecular Probes) that bind small molecules. Clearly, any fluorescent molecule detecting a small molecule can be used instead of the calcium fluorescent molecule, fluo-4 (Molecular Probes, Inc.; catalog no. F-14202), used here.

For adherent cells, seed the cells at 10,000 -20,000 cells/well in a Co-star black 96-well plate with clear bottom. The plate is incubated in a CO_2 incubator for 20 hours. The adherent cells are washed two times in Biotek washer with 200 ul of HBSS (Hank's Balanced Salt Solution) leaving 100 ul of buffer after the final wash.

A stock solution of 1 mg/ml fluo-4 is made in 10% pluronic acid DMSO. To load the cells with fluo-4, 50 ul of 12 ug/ml fluo-4 is added to each well. The plate is incubated at 37 degrees C in a CO_2 incubator for 60 min. The plate is washed four times in the Biotek washer with HBSS leaving 100 ul of buffer.

For non-adherent cells, the cells are spun down from culture media. Cells are re-suspended to 2-5x10^6 cells/ml with HBSS in a 50-ml conical tube. 4 ul of 1 mg/ml fluo-4 solution in 10% pluronic acid DMSO is added to each ml of cell suspension. The tube is then placed in a 37 degrees C water bath for 30-60 min. The cells are washed twice with HBSS, resuspended to 1x10^6 cells/ml, and dispensed into a microplate, 100 ul/well. The plate is centrifuged at 1000 rpm for 5 min. The plate is then washed once in Denley Cell Wash with 200 ul, followed by an aspiration step to 100 ul final volume.

For a non-cell based assay, each well contains a fluorescent molecule, such as fluo-4 . The fusion protein of the invention is added to the well, and a change in fluorescence is detected.

To measure the fluorescence of intracellular calcium, the FLIPR is set for the following parameters: (1) System gain is 300-800 mW; (2) Exposure time is 0.4 second; (3) Camera F/stop is F/2; (4) Excitation is 488 nm; (5) Emission is 530 nm; and (6) Sample addition is 50 ul. Increased emission at 530 nm indicates an extracellular signaling event caused by an albumin fusion protein of the present invention or a molecule induced by an albumin fusion protein of the present invention, which has resulted in an increase in the intracellular Ca^{++} concentration.

Example 35: Assay Identifying Tyrosine Kinase Activity

The Protein Tyrosine Kinases (PTK) represent a diverse group of transmembrane and cytoplasmic kinases. Within the Receptor Protein Tyrosine Kinase (RPTK) group are receptors for a range of mitogenic and metabolic growth factors including the PDGF, FGF, EGF, NGF, HGF and Insulin receptor subfamilies. In addition there are a large family of RPTKs for which the corresponding ligand is unknown. Ligands for RPTKs include mainly secreted small proteins, but also membrane-bound and extracellular matrix proteins.

Activation of RPTK by ligands involves ligand-mediated receptor dimerization, resulting in transphosphorylation of the receptor subunits and activation of the cytoplasmic tyrosine kinases. The cytoplasmic tyrosine kinases include receptor associated tyrosine kinases of the src-family (e.g., src, yes, lck, lyn, fyn) and non-receptor linked and cytosolic protein tyrosine kinases, such as the Jak family, members of which mediate signal transduction triggered by the cytokine superfamily of receptors (e.g., the Interleukins, Interferons, GM-CSF, and Leptin).

Because of the wide range of known factors capable of stimulating tyrosine kinase activity, identifying whether an albumin fusion protein of the present invention or a molecule induced by a fusion proetin of the present invention is capable of activating tyrosine kinase signal transduction pathways is of interest. Therefore, the following protocol is designed to identify such molecules capable of activating the tyrosine kinase signal transduction pathways.

Seed target cells (e.g., primary keratinocytes) at a density of approximately 25,000 cells per well in a 96 well Loprodyne Silent Screen Plates purchased from Nalge Nunc (Naperville, IL). The plates are sterilized with two 30 minute rinses with 100% ethanol, rinsed with water and dried overnight. Some plates are coated for 2 hr with 100 ml of cell culture grade type I collagen (50 mg/ml), gelatin (2%) or polylysine (50 mg/ml), all of which can be purchased from Sigma Chemicals (St. Louis, MO) or 10% Matrigel purchased from Becton Dickinson (Bedford,MA), or calf serum, rinsed with PBS and stored at 4 degree C. Cell growth on these plates is assayed by seeding 5,000 cells/well in growth medium and indirect quantitation of cell number through use of alamarBlue as described by the manufacturer Alamar Biosciences, Inc. (Sacramento, CA) after 48 hr. Falcon plate covers #3071 from Becton Dickinson (Bedford,MA) are used to cover the Loprodyne Silent Screen Plates. Falcon Microtest III cell culture plates can also be used in some proliferation experiments.

To prepare extracts, A431 cells are seeded onto the nylon membranes of Loprodyne plates (20,000/200ml/well) and cultured overnight in complete medium. Cells are quiesced by incubation in serum-free basal medium for 24 hr. After 5-20 minutes treatment with EGF (60ng/ml) or a different concentrations of an albumin fusion protein of the invention, the medium was removed and 100 ml of extraction buffer ((20 mM HEPES pH 7.5, 0.15 M NaCl, 1% Triton X-100, 0.1% SDS, 2 mM Na3VO4, 2 mM Na4P2O7 and a cocktail of protease inhibitors (# 1836170) obtained from Boeheringer Mannheim (Indianapolis, IN)) is added to each well and the plate is shaken on a rotating shaker for 5 minutes at 4°C. The plate is then placed in a vacuum transfer manifold and the extract filtered through the 0.45 mm membrane bottoms of each well using house vacuum. Extracts are collected in a 96-well

catch/assay plate in the bottom of the vacuum manifold and immediately placed on ice. To obtain extracts clarified by centrifugation, the content of each well, after detergent solubilization for 5 minutes, is removed and centrifuged for 15 minutes at 4 degree C at 16,000 x g.

Test the filtered extracts for levels of tyrosine kinase activity. Although many methods of detecting tyrosine kinase activity are known, one method is described here.

Generally, the tyrosine kinase activity of an albumin fusion protein of the invention is evaluated by determining its ability to phosphorylate a tyrosine residue on a specific substrate (a biotinylated peptide). Biotinylated peptides that can be used for this purpose include PSK1 (corresponding to amino acids 6-20 of the cell division kinase cdc2-p34) and PSK2 (corresponding to amino acids 1-17 of gastrin). Both peptides are substrates for a range of tyrosine kinases and are available from Boehringer Mannheim.

The tyrosine kinase reaction is set up by adding the following components in order. First, add 10ul of 5uM Biotinylated Peptide, then 10ul ATP/Mg_{2+} (5mM ATP/50mM $MgCl_2$), then 10ul of 5x Assay Buffer (40mM imidazole hydrochloride, pH7.3, 40 mM beta-glycerophosphate, 1mM EGTA, 100mM $MgCl_2$, 5 mM $MnCl_2$, 0.5 mg/ml BSA), then 5ul of Sodium Vanadate(1mM), and then 5ul of water. Mix the components gently and preincubate the reaction mix at 30 degree C for 2 min. Initial the reaction by adding 10ul of the control enzyme or the filtered supernatant.

The tyrosine kinase assay reaction is then terminated by adding 10 ul of 120mm EDTA and place the reactions on ice.

Tyrosine kinase activity is determined by transferring 50 ul aliquot of reaction mixture to a microtiter plate (MTP) module and incubating at 37 degree C for 20 min. This allows the streptavidin coated 96 well plate to associate with the biotinylated peptide. Wash the MTP module with 300ul/well of PBS four times. Next add 75 ul of anti-phospotyrosine antibody conjugated to horse radish peroxidase(anti-P-Tyr-POD(0.5u/ml)) to each well and incubate at 37 degree C for one hour. Wash the well as above.

Next add 100ul of peroxidase substrate solution (Boehringer Mannheim) and incubate at room temperature for at least 5 mins (up to 30 min). Measure the absorbance of the sample at 405 nm by using ELISA reader. The level of bound peroxidase activity is quantitated using an ELISA reader and reflects the level of tyrosine kinase activity.

Example 36: Assay Identifying Phosphorylation Activity

As a potential alternative and/or complement to the assay of protein tyrosine kinase activity described in Example 35, an assay which detects activation (phosphorylation) of major intracellular signal transduction intermediates can also be used. For example, as

described below one particular assay can detect tyrosine phosphorylation of the Erk-1 and Erk-2 kinases. However, phosphorylation of other molecules, such as Raf, JNK, p38 MAP, Map kinase kinase (MEK), MEK kinase, Src, Muscle specific kinase (MuSK), IRAK, Tec, and Janus, as well as any other phosphoserine, phosphotyrosine, or phosphothreonine molecule, can be detected by substituting these molecules for Erk-1 or Erk-2 in the following assay.

Specifically, assay plates are made by coating the wells of a 96-well ELISA plate with 0.1ml of protein G (1ug/ml) for 2 hr at room temp, (RT). The plates are then rinsed with PBS and blocked with 3% BSA/PBS for 1 hr at RT. The protein G plates are then treated with 2 commercial monoclonal antibodies (100ng/well) against Erk-1 and Erk-2 (1 hr at RT) (Santa Cruz Biotechnology). (To detect other molecules, this step can easily be modified by substituting a monoclonal antibody detecting any of the above described molecules.) After 3-5 rinses with PBS, the plates are stored at 4 degree C until use.

A431 cells are seeded at 20,000/well in a 96-well Loprodyne filterplate and cultured overnight in growth medium. The cells are then starved for 48 hr in basal medium (DMEM) and then treated with EGF (6ng/well) or varying concentrations of the fusion protein of the invention for 5-20 minutes. The cells are then solubilized and extracts filtered directly into the assay plate.

After incubation with the extract for 1 hr at RT, the wells are again rinsed. As a positive control, a commercial preparation of MAP kinase (10ng/well) is used in place of A431 extract. Plates are then treated with a commercial polyclonal (rabbit) antibody (1ug/ml) which specifically recognizes the phosphorylated epitope of the Erk-1 and Erk-2 kinases (1 hr at RT). This antibody is biotinylated by standard procedures. The bound polyclonal antibody is then quantitated by successive incubations with Europium-streptavidin and Europium fluorescence enhancing reagent in the Wallac DELFIA instrument (time-resolved fluorescence). An increased fluorescent signal over background indicates a phosphorylation by the fusion protein of the present invention or a molecule induced by an albumin fusion protein of the present invention.

Example 37: Assay for the Stimulation of Bone Marrow CD34+ Cell Proliferation

This assay is based on the ability of human CD34+ to proliferate in the presence of hematopoietic growth factors and evaluates the ability of fusion proteins of the inventon to stimulate proliferation of CD34+ cells.

It has been previously shown that most mature precursors will respond to only a single signal. More immature precursors require at least two signals to respond. Therefore,

to test the effect of fusion proteins of the invention on hematopoietic activity of a wide range of progenitor cells, the assay contains a given fusion protein of the invention in the presence or absence of hematopoietic growth factors. Isolated cells are cultured for 5 days in the presence of Stem Cell Factor (SCF) in combination with tested sample. SCF alone has a very limited effect on the proliferation of bone marrow (BM) cells, acting in such conditions only as a "survival" factor. However, combined with any factor exhibiting stimulatory effect on these cells (e.g., IL-3), SCF will cause a synergistic effect. Therefore, if the tested fusion protein has a stimulatory effect on hematopoietic progenitors, such activity can be easily detected. Since normal BM cells have a low level of cycling cells, it is likely that any inhibitory effect of a given fusion protein might not be detected. Accordingly, assays for an inhibitory effect on progenitors is preferably tested in cells that are first subjected to *in vitro* stimulation with SCF+IL+3, and then contacted with the compound that is being evaluated for inhibition of such induced proliferation.

Briefly, CD34+ cells are isolated using methods known in the art. The cells are thawed and resuspended in medium (QBSF 60 serum-free medium with 1% L-glutamine (500ml) Quality Biological, Inc., Gaithersburg, MD Cat# 160-204-101). After several gentle centrifugation steps at 200 x g, cells are allowed to rest for one hour. The cell count is adjusted to 2.5 x 10^5 cells/ml. During this time, 100 µl of sterile water is added to the peripheral wells of a 96-well plate. The cytokines that can be tested with an albumin fusion protein of the invention in this assay is rhSCF (R&D Systems, Minneapolis, MN, Cat# 255-SC) at 50 ng/ml alone and in combination with rhSCF and rhIL-3 (R&D Systems, Minneapolis, MN, Cat# 203-ML) at 30 ng/ml. After one hour, 10 µl of prepared cytokines, varying concentrations of an albumin fusion protein of the invention, and 20 µl of diluted cells are added to the media which is already present in the wells to allow for a final total volume of 100 µl. The plates are then placed in a 37°C/5% CO_2 incubator for five days.

Eighteen hours before the assay is harvested, 0.5 µCi/well of [3H] Thymidine is added in a 10 µl volume to each well to determine the proliferation rate. The experiment is terminated by harvesting the cells from each 96-well plate to a filtermat using the Tomtec Harvester 96. After harvesting, the filtermats are dried, trimmed and placed into OmniFilter assemblies consisting of one OmniFilter plate and one OmniFilter Tray. 60 µl Microscint is added to each well and the plate sealed with TopSeal-A press-on sealing film A bar code 15 sticker is affixed to the first plate for counting. The sealed plates are then loaded and the level of radioactivity determined via the Packard Top Count and the printed data collected for

analysis. The level of radioactivity reflects the amount of cell proliferation.

The studies described in this example test the activity of a given fusion protein to stimulate bone marrow CD34+ cell proliferation. One skilled in the art could easily modify the exemplified studies to test the activity of fusion porteins and polynucleotides of the invention (e.g., gene therapy) as well as agonists and antagonists thereof. The ability of an albumin fusion protein of the invention to stimulate the proliferation of bone marrow CD34+ cells indicates that the albumin fusion protein and/or polynucleotides corresponding to the fusion protein are useful for the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein.

Example 38: Assay for Extracellular Matrix Enhanced Cell Response (EMECR)

The objective of the Extracellular Matrix Enhanced Cell Response (EMECR) assay is to evaluate the ability of fusion proteins of the invention to act on hematopoietic stem cells in the context of the extracellular matrix (ECM) induced signal.

Cells respond to the regulatory factors in the context of signal(s) received from the surrounding microenvironment. For example, fibroblasts, and endothelial and epithelial stem cells fail to replicate in the absence of signals from the ECM. Hematopoietic stem cells can undergo self-renewal in the bone marrow, but not in *in vitro* suspension culture. The ability of stem cells to undergo self-renewal *in vitro* is dependent upon their interaction with the stromal cells and the ECM protein fibronectin (fn). Adhesion of cells to fn is mediated by the $\alpha_5.\beta_1$ and $\alpha_4.\beta_1$ integrin receptors, which are expressed by human and mouse hematopoietic stem cells. The factor(s) which integrate with the ECM environment and are responsible for stimulating stem cell self-renewal havea not yet been identified. Discovery of such factors should be of great interest in gene therapy and bone marrow transplant applications

Briefly, polystyrene, non tissue culture treated, 96-well plates are coated with fn fragment at a coating concentration of 0.2 μg/ cm^2. Mouse bone marrow cells are plated (1,000 cells/well) in 0.2 ml of serum-free medium. Cells cultured in the presence of IL-3 (5 ng/ml) + SCF (50 ng/ml) would serve as the positive control, conditions under which little self-renewal but pronounced differentiation of the stem cells is to be expected. Albumin fusion proteins of the invention are tested with appropriate negative controls in the presence and absence of SCF(5.0 ng/ml), where volume of the administed composition containing the albumin fusion protein of the invention represents 10% of the total assay volume. The plated cells are then allowed to grow by incubating in a low oxygen environment (5% CO_2, 7% O_2,

and 88% N_2) tissue culture incubator for 7 days. The number of proliferating cells within the wells is then quantitated by measuring thymidine incorporation into cellular DNA. Verification of the positive hits in the assay will require phenotypic characterization of the cells, which can be accomplished by scaling up of the culture system and using appropriate antibody reagents against cell surface antigens and FACScan.

One skilled in the art could easily modify the exemplified studies to test the activity of albumin fusion proteins and polynucleotides of the invention (e.g., gene therapy).

If a particular fusion protein of the present invention is found to be a stimulator of hematopoietic progenitors, the fusion protein and polynucleotides corresponding to the fusion protein may be useful for example, in the diagnosis and treatment of disorders affecting the immune system and hematopoiesis. Representative uses are described in the "Immune Activity" and "Infectious Disease" sections above, and elsewhere herein. The fusion protein may also be useful in the expansion of stem cells and committed progenitors of various blood lineages, and in the differentiation and/or proliferation of various cell types.

Additionally, the albumin fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention, may also be employed to inhibit the proliferation and differentiation of hematopoietic cells and therefore may be employed to protect bone marrow stem cells from chemotherapeutic agents during chemotherapy. This antiproliferative effect may allow administration of higher doses of chemotherapeutic agents and, therefore, more effective chemotherapeutic treatment.

Moreover, fusion proteins of the invention and polynucleotides encoding albumin fusion proteins of the invention may also be useful for the treatment and diagnosis of hematopoietic related disorders such as, anemia, pancytopenia, leukopenia, thrombocytopenia or leukemia, since stromal cells are important in the production of cells of hematopoietic lineages. The uses include bone marrow cell ex-vivo culture, bone marrow transplantation, bone marrow reconstitution, radiotherapy or chemotherapy of neoplasia.

Example 39: Human Dermal Fibroblast and Aortic Smooth Muscle Cell Proliferation

An albumin fusion protein of the invention is added to cultures of normal human dermal fibroblasts (NHDF) and human aortic smooth muscle cells (AoSMC) and two co-assays are performed with each sample. The first assay examines the effect of the fusion protein on the proliferation of normal human dermal fibroblasts (NHDF) or aortic smooth muscle cells (AoSMC). Aberrant growth of fibroblasts or smooth muscle cells is a part of several pathological processes, including fibrosis, and restenosis. The second assay examines IL6 production by both NHDF and SMC. IL6 production is an indication of

functional activation. Activated cells will have increased production of a number of cytokines and other factors, which can result in a proinflammatory or immunomodulatory outcome. Assays are run with and without co-TNFa stimulation, in order to check for costimulatory or inhibitory activity.

Briefly, on day 1, 96-well black plates are set up with 1000 cells/well (NHDF) or 2000 cells/well (AoSMC) in 100 µl culture media. NHDF culture media contains: Clonetics FB basal media, 1mg/ml hFGF, 5mg/ml insulin, 50mg/ml gentamycin, 2%FBS, while AoSMC culture media contains Clonetics SM basal media, 0.5 µg/ml hEGF, 5mg/ml insulin, 1µg/ml hFGF, 50mg/ml gentamycin, 50 µg/ml Amphotericin B, 5%FBS. After incubation at 37°C for at least 4-5 hours culture media is aspirated and replaced with growth arrest media. Growth arrest media for NHDF contains fibroblast basal media, 50mg/ml gentamycin, 2% FBS, while growth arrest media for AoSMC contains SM basal media, 50mg/ml gentamycin, 50µg/ml Amphotericin B, 0.4% FBS. Incubate at 37 °C until day 2.

On day 2, serial dilutions and templates of an albumin fusion protein of the invention are designed such that they always include media controls and known-protein controls. For both stimulation and inhibition experiments, proteins are diluted in growth arrest media. For inhibition experiments, TNFa is added to a final concentration of 2ng/ml (NHDF) or 5ng/ml (AoSMC). Add 1/3 vol media containing controls or an albumin fusion protein of the invention and incubate at 37 degrees C/5% CO_2 until day 5.

Transfer 60µl from each well to another labeled 96-well plate, cover with a plate-sealer, and store at 4 degrees C until Day 6 (for IL6 ELISA). To the remaining 100 µl in the cell culture plate, aseptically add Alamar Blue in an amount equal to 10% of the culture volume (10µl). Return plates to incubator for 3 to 4 hours. Then measure fluorescence with excitation at 530nm and emission at 590nm using the CytoFluor. This yields the growth stimulation/inhibition data.

On day 5, the IL6 ELISA is performed by coating a 96 well plate with 50-100 ul/well of Anti-Human IL6 Monoclonal antibody diluted in PBS, pH 7.4, incubate ON at room temperature.

On day 6, empty the plates into the sink and blot on paper towels. Prepare Assay Buffer containing PBS with 4% BSA. Block the plates with 200 µl/well of Pierce Super Block blocking buffer in PBS for 1-2 hr and then wash plates with wash buffer (PBS, 0.05% Tween-20). Blot plates on paper towels. Then add 50 µl/well of diluted Anti-Human IL-6 Monoclonal, Biotin-labeled antibody at 0.50 mg/ml. Make dilutions of IL-6 stock in media (30, 10, 3, 1, 0.3, 0 ng/ml). Add duplicate samples to top row of plate. Cover the plates and

incubate for 2 hours at RT on shaker.

Plates are washed with wash buffer and blotted on paper towels. Dilute EU-labeled Streptavidin 1:1000 in Assay buffer, and add 100 µl/well. Cover the plate and incubate 1 h at RT. Plates are again washed with wash buffer and blotted on paper towels.

Add 100 µl/well of Enhancement Solution. Shake for 5 minutes. Read the plate on the Wallac DELFIA Fluorometer. Readings from triplicate samples in each assay were tabulated and averaged.

A positive result in this assay suggests AoSMC cell proliferation and that the albumin fusion protein may be involved in dermal fibroblast proliferation and/or smooth muscle cell proliferation. A positive result also suggests many potential uses of the fusion protein and polynucleotides encoding the albumin fusion protein. For example, inflammation and immune responses, wound healing, and angiogenesis, as detailed throughout this specification. Particularly, fusion proteins may be used in wound healing and dermal regeneration, as well as the promotion of vasculogenesis, both of the blood vessels and lymphatics. The growth of vessels can be used in the treatment of, for example, cardiovascular diseases. Additionally, fusion proteins showing antagonistic activity in this assay may be useful in treating diseases, disorders, and/or conditions which involve angiogenesis by acting as an anti-vascular agent (e.g., anti-angiogenesis). These diseases, disorders, and/or conditions are known in the art and/or are described herein, such as, for example, malignancies, solid tumors, benign tumors, for example hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic granulomas; artheroscleric plaques; ocular angiogenic diseases, for example, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis, retinoblastoma, uvietis and Pterygia (abnormal blood vessel growth) of the eye; rheumatoid arthritis; psoriasis; delayed wound healing; endometriosis; vasculogenesis; granulations; hypertrophic scars (keloids); nonunion fractures; scleroderma; trachoma; vascular adhesions; myocardial angiogenesis; coronary collaterals; cerebral collaterals; arteriovenous malformations; ischemic limb angiogenesis; Osler-Webber Syndrome; plaque neovascularization; telangiectasia; hemophiliac joints; angiofibroma; fibromuscular dysplasia; wound granulation; Crohn's disease; and atherosclerosis. Moreover, albumin fusion proteins that act as antagonists in this assay may be useful in treating anti-hyperproliferative diseases and/or anti-inflammatory known in the art and/or described herein.

Example 40: Cellular Adhesion Molecule (CAM) Expression on Endothelial Cells

The recruitment of lymphocytes to areas of inflammation and angiogenesis involves

specific receptor-ligand interactions between cell surface adhesion molecules (CAMs) on lymphocytes and the vascular endothelium. The adhesion process, in both normal and pathological settings, follows a multi-step cascade that involves intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and endothelial leukocyte adhesion molecule-1 (E-selectin) expression on endothelial cells (EC). The expression of these molecules and others on the vascular endothelium determines the efficiency with which leukocytes may adhere to the local vasculature and extravasate into the local tissue during the development of an inflammatory response. The local concentration of cytokines and growth factor participate in the modulation of the expression of these CAMs.

Briefly, endothelial cells (e.g., Human Umbilical Vein Endothelial cells (HUVECs)) are grown in a standard 96 well plate to confluence, growth medium is removed from the cells and replaced with 100 μl of 199 Medium (10% fetal bovine serum (FBS)). Samples for testing (containing an albumin fusion protein of the invention) and positive or negative controls are added to the plate in triplicate (in 10 μl volumes). Plates are then incubated at 37°C for either 5 h (selectin and integrin expression) or 24 h (integrin expression only). Plates are aspirated to remove medium and 100 μl of 0.1% paraformaldehyde-PBS(with Ca++ and Mg++) is added to each well. Plates are held at 4°C for 30 min. Fixative is removed from the wells and wells are washed 1X with PBS(+Ca,Mg) + 0.5% BSA and drained. 10 μl of diluted primary antibody is added to the test and control wells. Anti-ICAM-1-Biotin, Anti-VCAM-1-Biotin and Anti-E-selectin-Biotin are used at a concentration of 10 μg/ml (1:10 dilution of 0.1 mg/ml stock antibody). Cells are incubated at 37°C for 30 min. in a humidified environment. Wells are washed three times with PBS(+Ca,Mg) + 0.5% BSA. 20 μl of diluted ExtrAvidin-Alkaline Phosphatase (1:5,000 dilution, referred to herein as the working dilution) are added to each well and incubated at 37°C for 30 min. Wells are washed three times with PBS(+Ca,Mg)+0.5% BSA. Dissolve 1 tablet of p-Nitrophenol Phosphate pNPP per 5 ml of glycine buffer (pH 10.4). 100 μl of pNPP substrate in glycine buffer is added to each test well. Standard wells in triplicate are prepared from the working dilution of the ExtrAvidin-Alkaline Phosphotase in glycine buffer: 1:5,000 (10^{0}) > $10^{-0.5}$ > 10^{-1} > $10^{-1.5}$. 5 μl of each dilution is added to triplicate wells and the resulting AP content in each well is 5.50 ng, 1.74 ng, 0.55 ng, 0.18 ng. 100 μl of pNNP reagent is then added to each of the standard wells. The plate is incubated at 37°C for 4h. A volume of 50 μl of 3M NaOH is added to all wells. The plate is read on a plate reader at 405 nm using the background subtraction option on blank wells filled with glycine buffer only. Additionally, the template is set up to indicate the concentration of AP-conjugate in each standard well [5.50 ng; 1.74 ng; 0.55 ng; 0.18 ng]. Results are indicated as amount of bound AP-conjugate in each sample.

Example 41: Alamar Blue Endothelial Cells Proliferation Assay

This assay may be used to quantitatively determine protein mediated inhibition of bFGF-induced proliferation of Bovine Lymphatic Endothelial Cells (LECs), Bovine Aortic Endothelial Cells (BAECs) or Human Microvascular Uterine Myometrial Cells (UTMECs). This assay incorporates a fluorometric growth indicator based on detection of metabolic activity. A standard Alamar Blue Proliferation Assay is prepared in EGM-2MV with 10 ng /ml of bFGF added as a source of endothelial cell stimulation. This assay may be used with a variety of endothelial cells with slight changes in growth medium and cell concentration. Dilutions of protein batches to be tested are diluted as appropriate. Serum-free medium (GIBCO SFM) without bFGF is used as a non-stimulated control and Angiostatin or TSP-1 are included as a known inhibitory controls.

Briefly, LEC, BAECs or UTMECs are seeded in growth media at a density of 5000 to 2000 cells/well in a 96 well plate and placed at 37 degreesC overnight. After the overnight incubation of the cells, the growth media is removed and replaced with GIBCO EC-SFM. The cells are treated with the appropriate dilutions of an albumin fusion protein of the invention or control protein sample(s) (prepared in SFM) in triplicate wells with additional bFGF to a concentration of 10 ng/ ml. Once the cells have been treated with the samples, the plate(s) is/are placed back in the 37° C incubator for three days. After three days 10 ml of stock alamar blue (Biosource Cat# DAL1100) is added to each well and the plate(s) is/are placed back in the 37°C incubator for four hours. The plate(s) are then read at 530nm excitation and 590nm emission using the CytoFluor fluorescence reader. Direct output is recorded in relative fluorescence units.

Alamar blue is an oxidation-reduction indicator that both fluoresces and changes color in response to chemical reduction of growth medium resulting from cell growth. As cells grow in culture, innate metabolic activity results in a chemical reduction of the immediate surrounding environment. Reduction related to growth causes the indicator to change from oxidized (non-fluorescent blue) form to reduced (fluorescent red) form (i.e., stimulated proliferation will produce a stronger signal and inhibited proliferation will produce a weaker signal and the total signal is proportional to the total number of cells as well as their metabolic activity). The background level of activity is observed with the starvation medium alone. This is compared to the output observed from the positive control samples (bFGF in growth medium) and protein dilutions.

Example 42: Detection of Inhibition of a Mixed Lymphocyte Reaction

This assay can be used to detect and evaluate inhibition of a Mixed Lymphocyte Reaction (MLR) by fusion proteins of the invention. Inhibition of a MLR may be due to a

direct effect on cell proliferation and viability, modulation of costimulatory molecules on interacting cells, modulation of adhesiveness between lymphocytes and accessory cells, or modulation of cytokine production by accessory cells. Multiple cells may be targeted by the albumin fusion proteins that inhibit MLR since the peripheral blood mononuclear fraction used in this assay includes T, B and natural killer lymphocytes, as well as monocytes and dendritic cells.

Albumin fusion proteins of the invention found to inhibit the MLR may find application in diseases associated with lymphocyte and monocyte activation or proliferation. These include, but are not limited to, diseases such as asthma, arthritis, diabetes, inflammatory skin conditions, psoriasis, eczema, systemic lupus erythematosus, multiple sclerosis, glomerulonephritis, inflammatory bowel disease, crohn's disease, ulcerative colitis, arteriosclerosis, cirrhosis, graft vs. host disease, host vs. graft disease, hepatitis, leukemia and lymphoma.

Briefly, PBMCs from human donors are purified by density gradient centrifugation using Lymphocyte Separation Medium (LSM®, density 1.0770 g/ml, Organon Teknika Corporation, West Chester, PA). PBMCs from two donors are adjusted to 2 x 10^6 cells/ml in RPMI-1640 (Life Technologies, Grand Island, NY) supplemented with 10% FCS and 2 mM glutamine. PBMCs from a third donor is adjusted to 2 x 10^5 cells/ml. Fifty microliters of PBMCs from each donor is added to wells of a 96-well round bottom microtiter plate. Dilutions of the fusion protein test material (50 µl) is added in triplicate to microtiter wells. Test samples (of the protein of interest) are added for final dilution of 1:4; rhuIL-2 (R&D Systems, Minneapolis, MN, catalog number 202-IL) is added to a final concentration of 1 µg/ml; anti-CD4 mAb (R&D Systems, clone 34930.11, catalog number MAB379) is added to a final concentration of 10 µg/ml. Cells are cultured for 7-8 days at 37°C in 5% CO_2, and 1 µC of [^{3}H] thymidine is added to wells for the last 16 hrs of culture. Cells are harvested and thymidine incorporation determined using a Packard TopCount. Data is expressed as the mean and standard deviation of triplicate determinations.

Samples of the fusion protein of interest are screened in separate experiments and compared to the negative control treatment, anti-CD4 mAb, which inhibits proliferation of lymphocytes and the positive control treatment, IL-2 (either as recombinant material or supernatant), which enhances proliferation of lymphocytes.

Example 43: Assays for Protease Activity

The following assay may be used to assess protease activity of an albumin fusion

protein of the invention.

Gelatin and casein zymography are performed essentially as described (Heusen et al., *Anal. Biochem.*, 102:196-202 (1980); Wilson et al., *Journal of Urology*, 149:653-658 (1993)). Samples are run on 10% polyacryamide/0.1% SDS gels containing 1% gelain orcasein, soaked in 2.5% triton at room temperature for 1 hour, and in 0.1M glycine, pH 8.3 at 37°C 5 to 16 hours. After staining in amido black areas of proteolysis apear as clear areas agains the blue-black background. Trypsin (Sigma T8642) is used as a positive control.

Protease activity is also determined by monitoring the cleavage of n-a-benzoyl-L-arginine ethyl ester (BAEE) (Sigma B-4500. Reactions are set up in (25mM$NaPO_4$,1mM EDTA, and 1mM BAEE), pH 7.5. Samples are added and the change in adsorbance at 260nm is monitored on the Beckman DU-6 spectrophotometer in the time-drive mode. Trypsin is used as a positive control.

Additional assays based upon the release of acid-soluble peptides from casein or hemoglobin measured as adsorbance at 280 nm or colorimetrically using the Folin method are performed as described in Bergmeyer, et al., *Methods of Enzymatic Analysis*, 5 (1984). Other assays involve the solubilization of chromogenic substrates (Ward, *Applied Science*, 251-317 (1983)).

Example 44: Identifying Serine Protease Substrate Specificity

Methods known in the art or described herein may be used to determine the substrate specificity of the albumin fusion proteins of the present invention having serine protease activity. A preferred method of determining substrate specificity is by the use of positional scanning synthetic combinatorial libraries as described in GB 2 324 529 (incorporated herein in its entirety).

Example 45: Ligand Binding Assays

The following assay may be used to assess ligand binding activity of an albumin fusion protein of the invention.

Ligand binding assays provide a direct method for ascertaining receptor pharmacology and are adaptable to a high throughput format. The purified ligand for an albumin fusion protein of the invention is radiolabeled to high specific activity (50-2000 Ci/mmol) for binding studies. A determination is then made that the process of radiolabeling does not diminish the activity of the ligand towards the fusion protein. Assay conditions for buffers, ions, pH and other modulators such as nucleotides are optimized to establish a workable signal to noise ratio for both membrane and whole cell polypeptide sources. For these assays, specific polypeptide binding is defined as total associated radioactivity minus the radioactivity

measured in the presence of an excess of unlabeled competing ligand. Where possible, more than one competing ligand is used to define residual nonspecific binding.

Example 46: Functional Assay in Xenopus Oocytes

Capped RNA transcripts from linearized plasmid templates encoding an albumin fusion protein of the invention is synthesized in vitro with RNA polymerases in accordance with standard procedures. In vitro transcripts are suspended in water at a final concentration of 0.2 mg/mi. Ovarian lobes are removed from adult female toads, Stage V defolliculated oocytes are obtained, and RNA transcripts (10 ng/oocytc) are injected in a 50 nl bolus using a microinjection apparatus. Two electrode voltage clamps are used to measure the currents from individual *Xenopus oocytes* in response fusion protein and polypeptide agonist exposure. Recordings are made in Ca2+ free Barth's medium at room temperature. The Xenopus system can be used to screen known ligands and tissue/cell extracts for activating ligands.

Example 47: Microphysiometric Assays

Activation of a wide variety of secondary messenger systems results in extrusion of small amounts of acid from a cell. The acid formed is largely as a result of the increased metabolic activity required to fuel the intracellular signaling process. The pH changes in the media surrounding the cell are very small but are detectable by the CYTOSENSOR microphysiometer (Molecular Devices Ltd., Menlo Park, Calif.). The CYTOSENSOR is thus capable of detecting the ability of an albumin fusion protein of the invention to activate secondary messengers that are coupled to an energy utilizing intracellular signaling pathway.

Example 48: Extract/Cell Supernatant Screening

A large number of mammalian receptors exist for which there remains, as yet, no cognate activating ligand (agonist). Thus, active ligands for these receptors may not be included within the ligands banks as identified to date. Accordingly, the albumin fusion proteins of the invention can also be functionally screened (using calcium, cAMP, microphysiometer, oocyte electrophysiology, etc., functional screens) against tissue extracts to identify natural ligands for the Therapeutic protein portion and/or albumin protein portion of an albumin fusion protein of the invention. Extracts that produce positive functional responses can be sequentially subfractionated until an activating ligand is isolated and identified.

Example 49: ATP-binding assay

The following assay may be used to assess ATP-binding activity of fusion proteins of

the invention.

ATP-binding activity of an albumin fusion protein of the invention may be detected using the ATP-binding assay described in U.S. Patent 5,858,719, which is herein incorporated by reference in its entirety. Briefly, ATP-binding to an albumin fusion protein of the invention is measured via photoaffinity labeling with 8-azido-ATP in a competition assay. Reaction mixtures containing 1 mg/ml of ABC transport protein are incubated with varying concentrations of ATP, or the non-hydrolyzable ATP analog adenyl-5'-imidodiphosphate for 10 minutes at 4°C. A mixture of 8-azido-ATP (Sigma Chem. Corp., St. Louis, MO.) plus 8-azido-ATP (^{32}P-ATP) (5 mCi/μmol, ICN, Irvine CA.) is added to a final concentration of 100 μM and 0.5 ml aliquots are placed in the wells of a porcelain spot plate on ice. The plate is irradiated using a short wave 254 nm UV lamp at a distance of 2.5 cm from the plate for two one-minute intervals with a one-minute cooling interval in between. The reaction is stopped by addition of dithiothreitol to a final concentration of 2mM. The incubations are subjected to SDS-PAGE electrophoresis, dried, and autoradiographed. Protein bands corresponding to the albumin fusion proteins of the invention are excised, and the radioactivity quantified. A decrease in radioactivity with increasing ATP or adenly-5'-imidodiphosphate provides a measure of ATP affinity to the fusion protein.

Example 50: Phosphorylation Assay

In order to assay for phosphorylation activity of an albumin fusion protein of the invention, a phosphorylation assay as described in U.S. Patent 5,958,405 (which is herein incorporated by reference) is utilized. Briefly, phosphorylation activity may be measured by phosphorylation of a protein substrate using gamma-labeled ^{32}P-ATP and quantitation of the incorporated radioactivity using a gamma radioisotope counter. The fusion portein of the invention is incubated with the protein substrate, ^{32}P-ATP, and a kinase buffer. The ^{32}P incorporated into the substrate is then separated from free ^{32}P-ATP by electrophoresis, and the incorporated ^{32}P is counted and compared to a negative control. Radioactivity counts above the negative control are indicative of phosphorylation activity of the fusion protein.

Example 51: Detection of Phosphorylation Activity (Activation) of an Albumin Fusion Protein of the Invention in the Presence of Polypeptide Ligands

Methods known in the art or described herein may be used to determine the phosphorylation activity of an albumin fusion protein of the invention. A preferred method of

determining phosphorylation activity is by the use of the tyrosine phosphorylation assay as described in US 5,817,471 (incorporated herein by reference).

Example 52: Identification Of Signal Transduction Proteins That Interact With An albumin fusion protein Of The Present Invention

Albumin fusion proteins of the invention may serve as research tools for the identification, characterization and purification of signal transduction pathway proteins or receptor proteins. Briefly, a labeled fusion protein of the invention is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, an albumin fusion protein of the invention is covalently coupled to a chromatography column. Cell-free extract derived from putative target cells, such as carcinoma tissues, is passed over the column, and molecules with appropriate affinity bind to the albumin fusion protein. The protein complex is recovered from the column, dissociated, and the recovered molecule subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

Example 53: IL-6 Bioassay

A variety of assays are known in the art for testing the proliferative effects of an albumin fusion protein of the invention. For example, one such asssay is the IL-6 Bioassay as described by Marz *et al.* (*Proc. Natl. Acad. Sci., U.S.A., 95*:3251-56 (1998), which is herein incorporated by reference). After 68 hrs. at 37°C, the number of viable cells is measured by adding the tetrazolium salt thiazolyl blue (MTT) and incubating for a further 4 hrs. at 37°C. B9 cells are lysed by SDS and optical density is measured at 570 nm. Controls containing IL-6 (positive) and no cytokine (negative) are Briefly, IL-6 dependent B9 murine cells are washed three times in IL-6 free medium and plated at a concentration of 5,000 cells per well in 50 μl, and 50 μl of fusion protein of the invention is added. utilized. Enhanced proliferation in the test sample(s) (containing an albumin fusion protein of the invention) relative to the negative control is indicative of proliferative effects mediated by the fusion protein.

Example 54: Support of Chicken Embryo Neuron Survival

To test whether sympathetic neuronal cell viability is supported by an albumin fusion protein of the invention, the chicken embryo neuronal survival assay of Senaldi *et al* may be utilized (*Proc. Natl. Acad. Sci., U.S.A., 96*:11458-63 (1998), which is herein incorporated by reference). Briefly, motor and sympathetic neurons are isolated from chicken embryos,

resuspended in L15 medium (with 10% FCS, glucose, sodium selenite, progesterone, conalbumin, putrescine, and insulin; Life Technologies, Rockville, MD.) and Dulbecco's modified Eagles medium [with 10% FCS, glutamine, penicillin, and 25 mM Hepes buffer (pH 7.2); Life Technologies, Rockville, MD.], respectively, and incubated at 37°C in 5% CO_2 in the presence of different concentrations of the purified fusion protein of the invention, as well as a negative control lacking any cytokine. After 3 days, neuron survival is determined by evaluation of cellular morphology, and through the use of the colorimetric assay of Mosmann (Mosmann, T., *J. Immunol. Methods, 65*:55-63 (1983)). Enhanced neuronal cell viability as compared to the controls lacking cytokine is indicative of the ability of the albumin fusion protein to enhance the survival of neuronal cells.

Example 55: Assay for Phosphatase Activity

The following assay may be used to assess serine/threonine phosphatase (PTPase) activity of an albumin fusion protein of the invention.

In order to assay for serine/threonine phosphatase (PTPase) activity, assays can be utilized which are widely known to those skilled in the art. For example, the serine/threonine phosphatase (PSPase) activity of an albumin fusion protein of the invention may be measured using a PSPase assay kit from New England Biolabs, Inc. Myelin basic protein (MyBP), a substrate for PSPase, is phosphorylated on serine and threonine residues with cAMP-dependent Protein Kinase in the presence of [^{32}P]ATP. Protein serine/threonine phosphatase activity is then determined by measuring the release of inorganic phosphate from 32P-labeled MyBP.

Example 56: Interaction of Serine/Threonine Phosphatases with other Proteins

Fusion protein of the invention having serine/threonine phosphatase activity (e.g., as determined in Example 55) are useful, for example, as research tools for the identification, characterization and purification of additional interacting proteins or receptor proteins, or other signal transduction pathway proteins. Briefly, a labeled fusion protein of the invention is useful as a reagent for the purification of molecules with which it interacts. In one embodiment of affinity purification, an albumin fusion protein of the invention is covalently coupled to a chromatography column. Cell-free extract derived from putative target cells, such as neural or liver cells, is passed over the column, and molecules with appropriate affinity bind to the fusion protein. The fusion protein -complex is recovered from the column, dissociated, and the recovered molecule subjected to N-terminal protein sequencing. This amino acid sequence is then used to identify the captured molecule or to design degenerate

oligonucleotide probes for cloning the relevant gene from an appropriate cDNA library.

Example 57: Assaying for Heparanase Activity

There a numerous assays known in the art that may be employed to assay for heparanase activity of an albumin fusion protein of the invention. In one example, heparanase activity of an albumin fusion protein of the invention, is assayed as described by Vlodavsky et al., (Vlodavsky et al., Nat. Med., 5:793-802 (1999)). Briefly, cell lysates, conditioned media, intact cells (1 x 10^6 cells per 35-mm dish), cell culture supernatant, or purified fusion protein are incubated for 18 hrs at 37°C, pH 6.2-6.6, with ^{35}S-labeled ECM or soluble ECM derived peak I proteoglycans. The incubation medium is centrifuged and the supernatant is analyzed by gel filtration on a Sepharose CL-6B column (0.9 x 30 cm). Fractions are eluted with PBS and their radioactivity is measured. Degradation fragments of heparan sulfate side chains are eluted from Sepharose 6B at $0.5 < K_{av} < 0.8$ (peak II). Each experiment is done at least three times. Degradation fragments corresponding to "peak II," as described by Vlodavsky et al., is indicative of the activity of an albumin fusion protein of the invention in cleaving heparan sulfate.

Example 58: Immobilization of biomolecules

This example provides a method for the stabilization of an albumin fusion protein of the invention in non-host cell lipid bilayer constucts (see, e.g., Bieri et al., Nature Biotech 17:1105-1108 (1999), hereby incorporated by reference in its entirety herein) which can be adapted for the study of fusion proteins of the invention in the various functional assays described above. Briefly, carbohydrate-specific chemistry for biotinylation is used to confine a biotin tag to an albumin fusion protein of the invention, thus allowing uniform orientation upon immobilization. A 50uM solution of an albumin fusion protein of the invention in washed membranes is incubated with 20 mM NaIO4 and 1.5 mg/ml (4mM) BACH or 2 mg/ml (7.5mM) biotin-hydrazide for 1 hr at room temperature (reaction volume, 150ul). Then the sample is dialyzed (Pierce Slidealizer Cassett, 10 kDa cutoff; Pierce Chemical Co., Rockford IL) at 4C first for 5 h, exchanging the buffer after each hour, and finally for 12 h against 500 ml buffer R (0.15 M NaCl, 1 mM MgCl2, 10 mM sodium phosphate, pH7). Just before addition into a cuvette, the sample is diluted 1:5 in buffer ROG50 (Buffer R supplemented with 50 mM octylglucoside).

Example 59: Assays for Metalloproteinase Activity

Metalloproteinases are peptide hydrolases which use metal ions, such as Zn^{2+}, as the catalytic mechanism. Metalloproteinase activity of an albumin fusion protein of the present

invention can be assayed according to methods known in the art. The following exemplary methods are provided:

Proteolysis of alpha-2-macroglobulin

To confirm protease activity, a purified fusion protein of the invention is mixed with the substrate alpha-2-macroglobulin (0.2 unit/ml; Boehringer Mannheim, Germany) in 1x assay buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$ and 0.05% Brij-35) and incubated at 37°C for 1-5 days. Trypsin is used as positive control. Negative controls contain only alpha-2-macroglobulin in assay buffer. The samples are collected and boiled in SDS-PAGE sample buffer containing 5% 2-mercaptoethanol for 5-min, then loaded onto 8% SDS-polyacrylamide gel. After electrophoresis the proteins are visualized by silver staining. Proteolysis is evident by the appearance of lower molecular weight bands as compared to the negative control.

Inhibition of alpha-2-macroglobulin proteolysis by inhibitors of metalloproteinases

Known metalloproteinase inhibitors (metal chelators (EDTA, EGTA, AND $HgCl_2$), peptide metalloproteinase inhibitors (TIMP-1 and TIMP–2), and commercial small molecule MMP inhibitors) may also be used to characterize the proteolytic activity of an albumin fusion protein of the invention. Three synthetic MMP inhibitors that may be used are: MMP inhibitor I, [IC_{50} = 1.0 μM against MMP-1 and MMP–8; IC_{50} = 30 μM against MMP-9; IC_{50} = 150 μM against MMP-3]; MMP-3 (stromelysin-1) inhibitor I [IC_{50} = 5 μM against MMP-3], and MMP-3 inhibitor II [K_i = 130 nM against MMP-3]; inhibitors available through Calbiochem, catalog # 444250, 444218, and 444225, respectively). Briefly, different concentrations of the small molecule MMP inhibitors are mixed with a purified fusion protein of the invention (50μg/ml) in 22.9 μl of 1x HEPES buffer (50 mM HEPES, pH 7.5, 0.2 M NaCl, 10 mM $CaCl_2$, 25 μM $ZnCl_2$ and 0.05%Brij-35) and incubated at room temperature (24 °C) for 2-hr, then 7.1 μl of substrate alpha-2-macroglobulin (0.2 unit/ml) is added and incubated at 37°C for 20-hr. The reactions are stopped by adding 4x sample buffer and boiled immediately for 5 minutes. After SDS-PAGE, the protein bands are visualized by silver stain.

Synthetic Fluorogenic Peptide Substrates Cleavage Assay

The substrate specificity for fusion proteins of the invention with demonstrated metalloproteinase activity may be determined using techniques knonw in the art, such as using synthetic fluorogenic peptide substrates (purchased from BACHEM Bioscience Inc). Test substrates include, M-1985, M-2225, M-2105, M-2110, and M-2255. The first four are

MMP substrates and the last one is a substrate of tumor necrosis factor-α (TNF-α) converting enzyme (TACE). These substrastes are preferably prepared in 1:1 dimethyl sulfoxide (DMSO) and water. The stock solutions are 50-500 μM. Fluorescent assays are performed by using a Perkin Elmer LS 50B luminescence spectrometer equipped with a constant temperature water bath. The excitation λ is 328 nm and the emission λ is 393 nm. Briefly, the assay is carried out by incubating 176 μl 1x HEPES buffer (0.2 M NaCl, 10 mM $CaCl_2$, 0.05% Brij-35 and 50 mM HEPES, pH 7.5) with 4 μl of substrate solution (50 μM) at 25 °C for 15 minutes, and then adding 20 μl of a purified fusion protein of the invention into the assay cuvett. The final concentration of substrate is 1 μM. Initial hydrolysis rates are monitored for 30-min.

It will be clear that the invention may be practiced otherwise than as particularly described in the foregoing description and examples. Numerous modifications and variations of the present invention are possible in light of the above teachings and, therefore, are within the scope of the appended claims.

The entire disclosure of each document cited (including patents, patent applications, patent publications, journal articles, abstracts, laboratory manuals, books, or other disclosures) as well as information available through Identifiers specific to databases such as GenBank, GeneSeq, or the CAS Registry, referred to in this application are herein incorporated by reference in their entirety. The specification and sequence listing of each of the following U.S. applications are herein incorporated by reference in their entirety: Application Nos. 60/229,358 filed on April 12, 2000; 60/199,384 filed on April 25,2000 and 60/256,931 filed on December 21, 2000.

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CANADA

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NORWAY

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AUSTRALIA

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FINLAND

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SWEDEN

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